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Comptes rendus des séances de la Société de Biologie.
Séance du 27 Mai 1955. — Tome CXLIX, Novembre 1955, p. 2050

Inhibition de la multiplication du virus de la fièvre de la Vallée du Rift par le virus homologue irradié par des rayons ultraviolets.

Note de I. SAWA présentée par Y. NAGANO.

Ayant constaté que l'inactivation par des rayons ultraviolets du virus de la fièvre de la Vallée du Rift ne supprime pas son pouvoir antigénique, nous avons essayé de savoir le moment où s'établit l'immunité chez l'animal ayant reçu le virus irradié. A cet effet, nous avons commencé par la détermination : 1) du pouvoir du virus irradié à provoquer la production d'anticorps neutralisants, chez l'animal non réceptif ; 2) de l'effet inhibiteur sur l'infection, chez l'animal réceptif.

VIRUS. — Une souche apportée de l'Institut Pasteur de Paris en 1938. Le virus a été utilisé sous la forme du sérum de souris infectées recueilli peu avant la mort. La dose minima mortelle pour la souris a été de 0,25. 10^{-7}-10^{-8} cm^3.

IRRADIATION DU VIRUS. — Nous nous sommes servi d'une lampe de la Sankyo Electrique à vapeur de mercure à basse pression donnant 1,75 W pour la radiation 2.537 Å. Un centimètre cube de sérum virulent a été mis dans une boite de Petri d'un diamètre de 9 centimètres. La boite de Petri a été tenue à une distance de 13 centimètres de la lampe, et agitée à la main.

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Inactivation du virus par l'irradiation ultraviolette. — Le sérum virulent a subi une irradiation de durées variées : 3, 30, 60 secondes, 2, 5 ou 7 minutes. Le matériel irradié a été dilué à l'échelle décimale avec du bouillon et 0,25 cm^3 de chaque dilution a été injecté à la souris par voie sous-cutanée. Le résultat est montré dans le tableau I. Une irradiation de 3 secondes a fait tomber la virulence au centième. Le matériel irradié pendant 30 ou 60 secondes s'est montré non virulent. Mais, quand il a été dilué au dixième, il a tué la souris. L'irradiation de 2, 5 ou 7 minutes a atténué le virus remarquablement, mais n'a pas supprimé complètement la virulence.

Dilution du virus	Virus non irradié	Virus irradié pendant				
		3 secondes	30 secondes	60 secondes	2 minutes	5 minutes
non dilué..	+	+	—	—	+	+
10^1........	+	+	+	+	+	±
10^2........	+	+	+	+	±	—
10^3........	+	+	—	—	—	—
10^4........	+	+	—	—	—	—
10^5........	+	+	—	—	—	—
10^6........	+	—	—	—	—	—
10^7........	+	—	—	—	—	—
10^8........	+	—	—	—	—	—
10^9........	—	—	—	—	—	—

+ : virulent pour la Souris ;
— : non virulent.

Tableau I. — Inactivation du virus.

Protection de la souris par le virus irradié. — Si l'on injecte à la Souris le virus irradié pendant 30 secondes, l'animal résiste à l'inoculation d'épreuve effectuée quelques jours plus tard. En vue de préciser le moment d'établissement de la résistance, nous avons vacciné des souris par voie intraveineuse avec le sérum virulent irradié pendant 30 secondes à la dose de 0,4 cm^3. Au bout de 4, 3, 2, 1 jours ou immédiatement après vaccination, les animaux ont été éprouvés par l'inoculation intraveineuse de virus à une dose égale à $10^{2.0}$-$10^{2.5}$ fois la DL_{50}. Tous les animaux ont survécu, tandis que les témoins non vaccinés sont morts 28 à 40 heures après l'inoculation d'épreuve. Ces résultats nous indiquent que la résistance s'établit en 28 à 40 heures après la vaccination.

Inhibition de la multiplication du virus actif par le virus irradié. — Au bout de combien de temps après la vaccination, la multiplication du virus d'épreuve serait-elle inhibée ? Nous avons inoculé par voie intraveineuse un groupe de souris avec 0,4 cm^3 du virus irradié pendant 30 secondes. Des souris témoins ont reçu du sérum normal au lieu du vaccin irradié. Immédiatement après la vaccination, nous avons injecté par la même voie le virus actif à une dose égale $10^{2.0}$-$10^{2.5}$ fois la DL_{50}. Aux intervalles de 5, 15, 25 ou 45 heures, les

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animaux ont été saignés à blanc. On a soumis le foie au Waring-blendor avec du bouillon, foie par foie, pour préparer une suspension à 10 p. 100. On a centrifugé la suspension pendant 20 minutes à 3.000 tours. Le surnageant, dilué en série, a été injecté à la dose de 0,25 cm^3 sous la peau de souris neuves. Les résultats obtenus sont enregistrés dans le tableau II.

Inoculats	Virulence en log. de DL50 du foie au bout de			
	5 heures	15 heures	25 heures	45 heures
Virus irradié et virus actif		$<$1,0 $<$1,0 $<$1,0 $<$1,0 $<$1,0	$<$1,0 $<$1,0 $<$1,0 $<$1,0 $<$1,0	$<$1,0 $<$1,0 $<$1,0 $<$1,0 2,5
Témoin Sérum de souris neuve et virus actif	0,8 $<$1,0 $<$1.0 $<$1,0 $<$1.0	3,8 2,8 3,7 $<$5,0 $<$5,0	6,5 4,1 6,8 6,8 6,1	Les souris inoculées sont mortes

Tableau II. — Inhibition par le virus irradié de la multiplication du virus actif dans le foie.

Comme l'on voit dans le tableau, dans le foie de la souris non-vaccinée, la multiplication de virus commence vers la 15e heure et la quantité du virus augmente progressivement. Chez les souris vaccinées, la multiplication de virus n'a été décélée à aucun stade examiné.

C'est ainsi que nous avons constaté l'inhibition complète et précoce de la multiplication du virus. Pour élucider si les anticorps neutralisants interviennent dans cette inhibition, nous avons entrepris une série d'expériences dont les résultats seront exposés dans une note ultérieure.

Résumé. — 1) L'irradiation ultraviolette peut inactiver le virus de la fièvre de la Vallée du Rift en respectant son pouvoir antigénique. 2) Le virus irradié inhibe la multiplication du virus homologue actif. L'effet inhibiteur est complet et précoce.

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MONILIASIS

IMASATO DONOMAE & YŪZŌ KAWAMORI

The Third Internal Clinic, Medical School of Osaka University

(Received for publication September 28, 1955)

The incidence of moniliasis, which is caused by *Candida albicans*, in the realm of internal medicine is very rare in Japan.

Recently, by the courtesy of doctors in many of the clinics at large hospitals in this country, we were able to sum up cases of moniliasis experienced in the field of internal medicine since 1945. The authors will describe the clinical findings of these cases, and state the results of a few series experiments conducted by them especially concerning the mechanism of development of this disease.

I. Moniliasis Experienced in the Field of Internal Medicine in Japan.

CASTELLANI[1] reported the first case of moniliasis in the realm of internal medicine in 1905. This case was of bronchial origin. In Japan, Prof. MIKAMO[2] reported a case of pulmonary moniliasis in 1950, and the authors[3] also described a generalized moniliasis, which was ascertained by necropsy. The authors[4] experienced two more cases of pulmonary moniliasis which was diagnosed by biopsy of the resected lobe of the affected lung and by autopsy. Since then, reported cases of moniliasis in Japan have been increasing, totaling a number of 47. The types of these cases are indicated in Table 1.

Table 1. Internal moniliasis in Japan

	Primary	Secondary	Total
Generalized	6	5	11 (23.4%)
Respriratory	7	24	31 (66.0%)
Digestive	0	5	5 (10.6%)
Total	13 (28.3%)	34 (71.7%)	47 (100%)

As seen in the table, 47 cases consist of 31 in the respiratory tract, 11 generalized, and 5 in the digestive tract, and only 13 out of 47 were of primary infection. All the rest were secondary to other diseases showing in Table 2.

Table 2. Primary diseases of seccndary moniliasis

Type	Generalized	Respiratoric	Gigestic	Total
Number of cases	5	24	5	34
Pulm. Tbc.	0	5	0	5
Pulm. Tbc. of diabeticer	1	0	0	1
Tbc. meningitis	1	0	0	1
Pulm. Tbc. with intestinal Tbc.	0	1	0	1
Plum. gangrene and abscess	0	11	0	11
Pulm. cancer	0	3	0	3
Subacute endocarditis	0	1	0	1
Mitral stenosis	0	1	0	1
Collagen disease	0	1	0	1
Bacillary dysentery	1	0	1	2
Cholangitis and cholecystitis	0	0	4	4
Sepsis caused by pyocyaneus	1	0	0	1
Paratyphoid fever with diabetes	1	0	0	1
Undetermined	0	1	0	1

Two cases out of the 5 generalized and 1 out of the 24 respiratory occurred during the course of tuberculosis, 11 out of the respiratory occurred during pulmonary adscess or gangrene, and 4 out of the 5 secondary digestic moniliasis were sequences of cholangitis or cholecystitis. It is remarkable, that the primary diseases of secondary moniliasis were consumptive diseases.

Table 3 shows the sex and age distributions of the patients: over half of the total cases occur·ed after 41 years old, while no difference are detectable among female and male.

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Table 3. Sex and age distributions of internal moniliasis

Type	Sex		Age							Total
	M	F	−10	−20	−30	−40	−50	−60	−61	
Gen.	6	5	1	1	0	3	2	1	3	11
Res.	17	14	0	2	8	5	6	8	2	31
Dig.	4	1	0	0	0	1	0	3	1	5
Total	27	20	1	3	8	9	8	12	6	47
	57.4%	42.6%	4 8.5%		17 36.2%		26 55.3%			100%

The prognosis of these patients are given in Table 4, the lethality rate as high over 50%, especially 100% in generalized cases. Table 5 shows the time from onset to death of the cases. Although two third of the total cases died within 3 months after onset, there were 5 cases which took a course over 1 year.

Table 4. Prognosis of internal moniliasis

Type	Number of cases	Prognosis			
		Dead	Improved	Cured	Undetermined
Generalized	11	11	0	0	0
Respiratoric	31	11	8	11	1
Digestic	5	3	0	2	0
Total	47	25 (54.3%)	8 (17.4%)	13 (28.3%)	1

Table 5. The months until death

Type	−1 mos.	−3 mos.	−6 mos.	−1 yr.	−2 yrs.	2 yrs.−	Undetermined
Generalized	1	7	2	0	0	0	1
Respiratoric	1	3	2	2	1	1	1
Digestic	0	0	0	0	1	1	1
Total	2	10	4	2	2	2	3

Table 6. Diagnostic grounds of the internal moniliasis

Detection of *C. albicans*	Dignostic grounds			Type			
	Symptoms and signs	Ineffective antibiotic therapy	Autopsy or biopsy	Gen.	Resp.	Dig.	Total
0	0	0	0	5	9	0	14
Not exam.	0	0	0	2	2	0	4
0	0	0	—	2	0	0	2
0	0	0	Not exam.	2	17	5	24
	Undetermined			0	3	0	3
Total				11	31	5	47

As seen in Table 6, 14 cases were diagnosed on sure ground, they were; the detection of *Candida albicans* from the pathological material of patients, the various clinical symptoms and signs, no responsiveness against many antibiotic therapy, and, the assurance of diagnosis on autopsy or biopsy. Four more cases were diagnosed after autopsy in spite of the failure of the detection of *C. albicans* while living.

The symptoms of moniliasis are of various kinds, the symptoms and signs characteristic of internal moniliasis seen in this country are shown in Table 7. The most significant symptom is fever, high in 24 cases and slight in 15. All respiratory cases complained of cough with expectoration and rales in the chest were audible, most of the cases had abnormal roentgenogram.

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Table 7. Symptoms and signs of the internal moniliasis

Type		Gen.	Resp.	Dig.	Total
Number of cases		11	31	5	47
General symptoms	Fever high	9	12	3	24
	slightly	1	13	1	15
	Dyspnea	5	6	0	11
	Eruption, pustule	4	3	0	7
	Janudice	1	0	4	5
In chest	Cough and sputum	5	31	1	37
	Bloody sputum	1	5	0	6
	Chest pain	1	8	0	9
	Rales in chest	6	21	0	27
	Abnormality in chest roentgenogram	5	26	0	31
In abdomen	Nausea and vomiting	3	0	1	4
	Diarrhea	0	1	4	5
	Swollen liver	7	9	4	20
	Splenomegaly	3	1	1	5
	Swollen kidney	1	0	0	1
	Meteorism	3	2	0	5
	Abdominal pain	3	1	4	8

In the abdomen, the swollen livers were palpable in 20 cases, not only of digestic moniliasis but also of generalized and respiratoric cases. It is already well known that patients with moniliasis have various symptoms in the mouth. Our summarized results are as follows:

As seen in Table 8, so called "beef like" glossitis were seen in 20 out of 38 cases with the description of findings of tongues. In the oral cavities, there was no distinct signs except 6 thrush cases and 5 angular stomatities.

That sputum of moniliasis patients have a peculiar gelatinoid appearance is already well known. In our summarized cases, although only 8 cases were described about the character of their sputum, 5 of them had such gelatinoid sputa.

The chest roentgenograms of respiratory moniliasis are divided in two types; bronchial moniliasis having bronchitic shadows and pulmonary moniliasis having pneumonic or abscess shadows. In 6 cases of primary respiratoric moniliasis 2 showed roentgenograms of chronic bronchitic, 2 bronchopneumonic, 1 lobar pneumonic, and the other 2 of pulmonary abscesses.

As shown in Table 9, anemia is a common symptom in moniliasis, and leucocytosis is also seen in 8 cases, especially 5 cases of over 1,2000/cmm.

Table 8. Symptoms in oral cavities of moniliasis patients

Type		Gen.	Resp.	Dig.	Total
Number of cases		11	31	5	47
Tongue	beef-like	8	9	3	20 (52.6%)
	normal	2	15	1	18 (47.4%)
	not described	1	7	1	9
	furred	7	16	3	26 (63.4%)
	not furred	3	11	1	15 (36 6%)
	not described	1	4	1	6
Mucosa of oral cavity	reddened	4	0	0	6 (15.8%)
	icteric	0	0	2	2 (5.3%)
	thrush	3	2	1	6 (15.8%)
	normal	2	21	1	24 (63.1%)
	not described	2	6	1	9
Angular	stomatitis detectable	1	4	0	5 (12.0%)
	not detectable	9	24	4	37 (88.0%)
	not described	1	3	1	5

II. Diagnosis of Moniliasis, Especially Isolation of *Candida albicans*

As described in the previous chapter, there is no specific symptoms of moniliasis. The high fever, cough and sputum, especially gelatinoid expectration, and beef-like glossitis are suggestive signs for moniliasis but not decisive. And the invalidity of the antibiotic treatment is no more than the supportive means for diagnosis.

As the etiological agent of moniliasis is *Candida albicans*, the constant and abundant isolation of this organisms must be the important diagnostic means. But *C. albicans* are detectable from the saliva and feces of the healthy persons in the appropriate percentages, so the successful isolation of

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C. albicans does not mean the definitive requirement for establishing the diagnosis. Our results of the detection of *C. albicans* from the healthy persons and the tuberculous patients are as in Table 10.

As seen in Table 10, the authors could isolate *Candida* in 20~30% from the saliva or feces of the healthy doctors and nurses, but in only 3 or 4 cases by the three times repeated examinations. Contrary to this results, from the tuberculous patients in the same hospital, the authors could detect this organism in high rate, *i. e.* about 11% in sputum, 30% in saliva and 30% in feces. No *Candida* was isolated from the urine of the healthy persons.

Table 9. Hemogram of primary moniliasis

Type		Generalized	Respiratory	Total
Number of cases		5	7	12
Red cell	2~3 million	2	0	2
	3~4 million	1	5	6
	over 4 million	1	2	3
	not examined	1	0	1
White cell	4,000~6,000	1	0	1
	6,000~8,000	0	3	3
	8,000~12,000	1	2	3
	over 12,000	3	2	5
Neutrophil	under 60%	0	1	1
	60~70%	1	0	1
	70~80%	0	5	5
	over 80%	4	1	5
Lymphocyt	under 10%	2	1	3
	10~20%	2	4	6
	20~30%	1	2	3

Table 10. The detection of *C. alb.* from the healthy persons and non-moniliatic tuberculous patients

Material		Number of Cases	Detection of *C. albicans*: Negative	Positive: once	twice	thrice
Healthy	Saliva	60	48 (80.0%)	12 (20.0%)	5 (8.3%)	3 (5.0%)
	Feces	60	42 (70.0%)	18 (30.0%)	7 (11.5%)	4 (6.7%)
	Urine	60	60 (100%)	0	—	—
Tbc. patients	Saliva	817	495 (60.6%)	322 (39.4%)	258 (31.5%)	241 (29.5%)
	Sputum	817	638 (78.1%)	179 (21.9%)	108 (13.2%)	88 (10.8%)
	Feces	817	369 (45.2%)	448 (54.8%)	300 (36.7%)	242 (29.6%)

Table 11 shows the results of the detection of *Candida* from the mucous membranes and the surfaces of the teeth in the oral cavities of tuberculous patients, from whose saliva *Candida* organisms were cultured.

Table 11. The distribution of *Candida* group in the oral cavities

Examined locus	Number of examined cases	Positive cases
Gum	80	13 (16.3%)
Buccal mucosa	80	41 (51.3%)
Fur on the tongue	80	67 (84.5%)
Carious teeth	51	37 (72.5%)
Intact teeth	29	8 (27.6%)

Table 12. The detection of *Candida* from the various portions in the respiratoric tract of the patients *Candida* positive in sputum or saliva

Examined locus or materials	Examined cases	Positive cases
Pharynx	80	44 (55.0%)
Larynx	80	22 (27.5%)
Trachea	80	3 (3.8%)
Bifurcation	80	1 (1.3%)
Both main bronchuses	80	0
Bronchial lavages	101	0

The authors could isolate *Candida* organisms in very high rate from the fur on the tongue and the cavities of the carious teeth, *i. e.* 84.5% from the former and 72.5% from the latter.

As these results, there are the saprophytic *Candida* in the oral cavities of the healthy and non-moniliatic illed persons, so the detection of *C. albicans* in the sputum has only limited diagnostic value for moniliasis. The authors examined the secretes from the lower parts of the respiratory tracts of the *Candida*-in sputum patients by the JACKSON's bronchoscopic apparatus.

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As seen in Table 12, the rate of the isolation of *Candida* from the lower parts of the trachea was very low, no positive cases were observed in the main bronchus and bronchial lavage. From these results, the authors know that *Candida* is not present as saprophytic in the lower parts of the trachea and bronchus even if abundant in the saliva or sputnm. So the cultural examination of *Candida* from the secretes of the trachea or the bronchus must be valuable for differential diagnosis of respiratoric moniliasis.

Table 13 shows the results of the culture as to the material gained from the lower parts of the respiratory tracts of the moniliasis suspected patients. All five cases listed in Table 13 complained of productive cough and *Candida* body were isolated from their sputa, but in 4 cases the cultures of the secretions from tracheae and main bronchus gained by bronchoscopy were free from *Candida* group, these cases were finally diagnosed non-moniliatic diseases. In the last case in the table, *Candida albicans* were cultured from the trachea and main bronchus, the patient was diagnosed after clinical

Table 13. Detection of *C. albicans* from the bronchial secretions of the moniliasis suspected patients

Name of patients	Symptoms	*C. albicans* in sputum	*C. albicans* in bronchoscopically gained material		Final diagnosis
			Traches	Main bronchus	
MATSUOKA	Fever, cough & sputum	abundant	neg.	neg.	Pyothorax
YAMAMOTO	Fever, cough & sputum	abundant	neg.	neg.	Pulm. gangrene
ADACHI	Fever, cough & sputum	scanty	neg	neg.	Bronchial Tbc.
KOBASHI	Cough & sputum	abundant	neg.	neg.	Pulm. Tbc.
SHUIN	Fever, cough & sputum	abundant	moderately positive	scanty positive	Pulm. moniliasis

observations, as pulmonary moniliasis complicated by pyothorax mix-infected with *Pseudomonas aeruginosa*. Therefore, the bronchoscopy are useful procedure to exclude the final possibility of respiratoric moniliasis unfairly diagnosed.

The authors could conform the same relation of *Candida* in the feces and in the bile, for 4 out of 5 reported digestic monilia cases were the consequences of cholangitis or cholecystitis and *C. albicans* were isolated from the bile in three cases and from the C bile in another case, although in 10 healthy persons with positive *Candida* saliva and feces only one case was found positive for *Candida* in the bile.

The immuno-serological reaction has practically not useful for moniliasis, because from our studies of the agglutination, precipitation, complement fixation and intracutaneous reaction, we could not find any specific and characteristic differences between the moniliasis patients and healthy *Candida* excreters.

III. Mechanism of Moniliasis Development

No clear interpretations are yet presented as to the mechanism how moniliasis will develop by the saprophytic *Candida albicans*.

Some experiments have been undetaken to clarify this problem. In the first place, the authors examined the pathogenicity of the asprophytic strains of *Candida albicans* isolated from the healthy secreter by the intraperitoneal injection in the mice (strain NA 2), and compared them to those from moniliasis patients. Our results showed that, although the saprophytic strains were less virulent than those from the moniliasis patients, the former gained virulence as the latter after passages in the mice. The authors believe therefore that the saproyhytic *Candida albicans* strains must have the potential possibility to develop moniliasis.

It was interesting to be evidenced that the persons who have had carious teeth or other oral affection revealed much difference as (Antigen: 0.1 of the polysaccharide fraction of *Candida albicans*.) compared to healthy person in their intracutaneous reaction by the polysaccharide antigen.

Table 14 shows the results obtained by the polysaccharide antigen prepared by heating extraction in the acidic media; the positive reaction was decided by redness over 10 mm and suspicious by 5 to 9 mm after 48 hours after injection. As seen in the table, the positive reactors among the persons without illed teeth were under 10% and only 16% with alveolar pyorrhea, whereas, over half the cases out of 62 with carious teeth reacted positively to the intracutaneous injection of the polysaccharide

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Table 14. The intracutaneous reaction and the illed conditions in the oral cavities of the candida secreter

Oral condition	Examined cases	Intracutaneous reaction		
		negative	suspicious	positive
Carious teeth	62	22	7	33 (53%)
Carious teeth with alveolar pyorrhea	12	6	1	5 (42%)
Alveolar pyorrhea	32	21	6	5 (16%)
No illed signs	33	24	6	3 (9%)

antigens. (Antigen: 0.1 of the polysaccharide fraction of *Candida albicans*).

The authors suppose that the candida in the carious teeth may be not saprophytic but in inapparently infected condition. Whether the moniliasis develops or not under these conditions, must be decided by the virulence of *Candida albicans* and the resistance of the host. In the cases of moniliasis experienced in our country, this desease occurred more frequent in old age, or after chronic illness with emaciation as tuberculosis or diabetes.

In the experiments with mice, it was successfully demonstrated that the X-irradiated, alloxan diabetic, oxythiamin feeding or other bad conditioned animals died in higher rate and more quickly than the control untreated.

A number of investigators have implied that the antibiotic drugs are causative agents in the genesis of these mycotic infections. All 47 cases summarized by the authors were treated by various antibiotics before the development of moniliasis, 26 out of them by the combined therapy including penicillin, streptomycin, chlortetracycline, tetracycline, chloramphenicol, and erythromycin. Table 15 shows the influences of the antibiotic therapy on the course of moniliasis judged by each of the reported doctors.

Table 15. The influence of the antibiotic therapy against the course of moniliasis in Japan.

Type	Number of cases	Influences			
		provocative	worsend	initially improved then worsend	undetermined
Generalized	11	4	5	1	1
Respiratory	31	13	8	6	4
Digestive	5	2	1	1	1
Total	47	19 (46.3%)	14 (34.1%)	8 (19.5%)	6

As seen in this table, all cases except the definitively undetermined were influenced unfavorably by the antibiotic therapy, particularly 19 cases (46%) were determined by their reporters that they were provocated by the administration of the antibiotics. From these clinical facts, it would be impressively considered that the antibiotic therapy are one of the contributing factors to stimulate the development of moniliasis. A series of experiment has performed in order to attempt a clarification of this important problem.

Our results in regard to the influences of antibiotics on the growth or the virulence of *Candida albicans* is confirmed by many previous reports, that antibiotics neither stimulate the growth nor enhances the virulence of *Candida albicans*. However, a new and unknown fact was observed in tne xperiment which was performed regarding the accumulation of glutamic acid in the body of *Candida*. From the *Candida* body, which was incubated for one hour in a solution of glutamic acid at 37.5°C, free glutamic acid has been released after boiling for 30 minutes, and combined glutamic acid after hydrolysis in 5-N HCl for 10 hours. These released glutamic acid was quantitatively measured by the decarboxylases gained from coli bacilli. The glutamic acid in the *Candida* body did not increase when incubated in the solution containing only glutamic acid, but increased the free glutamic acid about 50% when incubated in the glutamic acid solution to which glucose was added.

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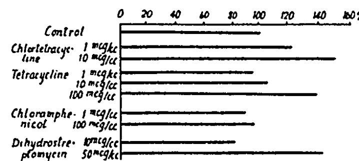
Fig. 1 shows the influences of the antibiotics upon the increase of the free glutamic acid in the *Candida* body under these conditions. It is clear that 10 mcg/cc of chlortetracycline (aureomycin) and 50 mcg/cc of dihydrostreptomycin promote the increase. Furthermore, the experiment respecting the increase of the free and combined glutamic acid, has brought a fact that the addition of 100 mcg/cc of chlortetracycline did enhance the accumulation of the combined type.

Fig. 1. The influences of antibiotics upon the increase of glutamic acid in *Candida albicans*. The rate of increase of the free glutamic acid in *Candida* body after 1-hour incubation at 37°C in glutamic acid solution.



The authors examined the influences of the antibiotics against the increase of the intracellular free and combined glutamic acid of the *Candida albicans* incubated in a mixture of various amino acids, and recognized that the increase was promoted by the addition of chlortetracycline in concentration of 100 mcg/cc. From these data, it may be highly suggestive that antibiotics of the tetracycline group are inhibitively influential to the protein synthesis in the *Candida* body. HUIPER and his collaborates[5] reported the increase of nitrogen in the *Candida* body by the addition of chlortetracycline in the cluture in spite of no increase of the cell counts. The authors consider that our results are related to their experiment.

SELIGMANN recently demonstrated in animal studies that sublethal dosis of *Candida albicans* became highly lethal when mixed with chlortetracycline. As shown in the Fig. 2, the mice that were infected

Fig. 2. The death rate of the mice infected with *Candida albicans* (30×10⁶) by simultaneous intraperitoneal injection of 1 mg chlortetracycline and tetracycline

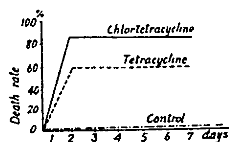


Fig. 3. The death rate of the *Candida*-infected mice with the administration of tetracycline at various time relating the infection

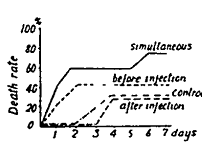
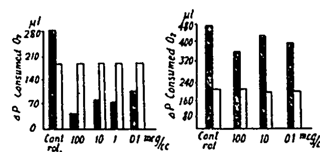


Fig. 4. The influence of the antibiotics against the oxidative phosphorylation



intraperitoneally by a combination of 1 mg of chlortetracycline or tetracycline (achromycin) died by 80% and 60% after 2 days respectively, though the control group without antibiotics survived completely even after 1 week.

As to the time of the administration of antibiotics, the authors performed research by the following experiment. Fig. 3 shows that if antibiotic is given simultaneously or pre-inflectious the influence against the death rate is higher than the post inflectious. In this respect, SELIGMANN[6] states that the administration of the antibiotics may bring about a lowered resistance to the host, not affecting the *Candida* body. Following are some interesting results that were obtained in researching this problems.

That the antibiotic may unfavorably inhibit the metabolism of vitamin B_1 and B_2 in the host, has been reported by Prof. AOYAMA[7] and Dr. KUBO[8]. For the purpose of clarifing this problem, the authors studied the effect of chlortetracycline and dehydrostreptomycin upon the oxidative phosphorylation using the liver of rat.

The results are shown in Fig. 4: The important points of this experimental methods are as follows: R 3 as the enzymatic material, hexokinase extracted from the bread yeast, and the estimation of the inorganic phosphor with the original method by FISKE. As seen in the figure, the addition of chlortetracycline in the emulsion of the rat liver markedly inhibited the decrease of inorganic phosphor but not the consumption of oxygen, although the dihydrostreptomycin was not affected.

Next researches were done on the phagocytosis in the host cell after infection by *Candida albicans*, injurious effect of antibiotics against them were detected. Fig. 5 compares the phagocytic rates in the cells gained from the ascitic fluid of the two groups of the mice infected intraperitoneally by *Candida* with and without simultaneous injection of tetracycline. The percentages in the figure shows

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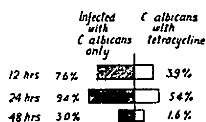
the average in five mice of each group. The phagocytic rate of the group administered with tetracycline are lower than these of the control group without that at 12, 24 and 48 hours after infection.

The authors examined also the number of the *Candida* in the ascites by the quantitative culture, and recognized that the *Candida* bodies were significantly abundant in the tetracycline combined mice. From these data, SELIGMAN's results concerning the increased death of mice infected by *Candida albicans* combined with antibiotics should be due to sepsis which occurred earlier and more frequently in the mice phagocytic activity were injured by the antibiotics.

Fig. 5. The phagocytic rate of the cells in the ascites of the mice intraperitoneally infected with *Candida albicans*



To ensure this point, experiments with the developing preparates of the subcutaneous connective tissue of the infected rabbit were performed. The preparations were gained by developing the subcutaneous connective tissue from the rabbits at 2 hours to 7 days after infection with *Candida*, and supravitally stained with P. A. S. method. The results in Fig. 6 shows that the phagocytic rate in the cell, especially in the monocytes, in the preparates from the rabbits to which 250 mg of chlortetracycline was given orally during 1 week before the infection were markedly lower than those of the animals without antibiotics.

However, the oral administration of chlortetracycline interferred the appetite of the rabbits significantly, so it was considered, that the general weakness caused by anorexia provoked the decreased phagocytosis. Our next experiment were to see if the same results were attained by infection the rabbits intraperitoneally with 50 mg of chlortetracyline for 7 days as to those administrated orally. The Fig. 7

Fig. 6. The phagocytic rate against *Candida albicans* in the developing preparates of the subcutaneous connective tissues from the rabbits infected subdermally with *Candida albicans*.

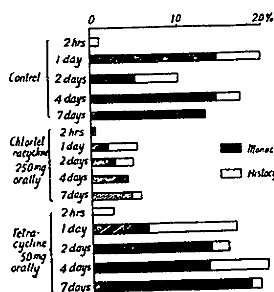
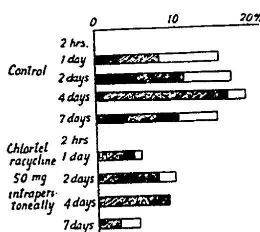


Fig. 7. The phagocytic rate against *Candida albicans* in the developing preparates of the subcutaneous connective tissues from the rabbits infected subdermally with *Candida albicans*



showes the marked decrease of phagocytic rate. From these experiments the authors were able to make clear the parts of the mechanisms through which moniliasis develops. concerning the inhibiting effects of the antibiotics against phagocytosis of the host.

Furthermore, some investigators stated that the alteration of the bacterial flora in the host treated by the antibiotic therapy should be a cause of the development of moniliasis. In the cases of moniliasis experienced in this country, various mixed infected bacteria were detected from many pathological materials as shown in Table 16.

Since in these cases included the secondary cases following other infections, the bacterial flora in this table might not be only a consequence of alteration. However, 2 of cases pyocyaneus bacilli (*Pseudomonas aeruginosa*) remained, surely, with *Candida albicans* after antibiotic therapy by the high resistance to those drugs.

The mechanism by which the alteration of bacterial flora promotes the development of the moniliasis is explained by most of the investigators that the flora with the competitive ability to *Candida albicans* are reduced by antibiotic therapy. The authors consider that under these conception, the relation between the alteration of flora and moniliasis, is not sufficiently explained. So the following experiments

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in regard to the mixed infection of the some bacteria with *Candida albicans* were performed by the authors.

Table 16. The mixed flora detected from the pathological material of moniliasis patients

Type Material	Generalized			Respiratory			Digestive	
	Blood	Sputum	C.S. fluid	Sputum	Cavity	Pleural	Bile fl.	Total
Staphylococcus	0	1	0	5	2	0	2	10
Pneumococcus	0	0	0	2	0	0	0	2
Streptococcus	0	0	0	5	2	0	0	7
Neisseria	0	2	0	1	0	0	0	3
Mycob. tuberculosis	0	1	1	2	0	0	0	4
Ps. aeruginosa	1	0	0	0	0	1	0	2
Escherichia coli	0	0	0	0	0	0	1	1
Aerob. aerogenes	0	0	0	1	0	0	0	1
Paracoli	0	0	0	2	0	0	0	2
Salm. paratyphi A	1	0	0	0	0	0	0	1
Miscellaneous bac.	0	1	0	3	2	0	2	8

The simultaneous injection of 1/10 M.L.D. of *Staphylococcus*, *Streptococcus hemolyticus*, *E. coli*, or *B. subtilis* could not influence the death rate of the mice infected with *Candida albicans*, whereas those of *Pseudomonas aeruginosa* or *Proteus vulgaris* have caused the high elevation of the death rate and the shortening of the survival time, as shown in Figs. 8 and 9.

Although only 20 to 40% of the mices infected by *Candida albicans* died within one week, those infected with *Proteus* or *Pseudomonas* dies at the rate of 100% within 3 to 4 days after infections, even with 1/20 M. L. D. of these bacteria.

Fig. 8. The death rate of the mice infected with *Candida albicans* and *Proteus vulgaris*

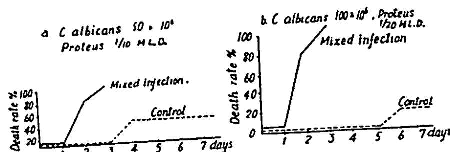
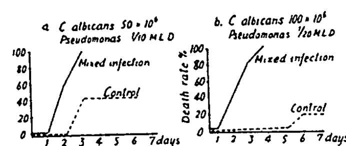


Fig. 9. The death rate of the mice infected with *Candida albicans* and *Pseudomonas aeruginosa*



The authors could detect both the organism, *i. e.* *Candida albicans* and *Proteus* or *Pseudomonas*, from the organs of those dead mice. So, which infection promoted the other was not decided. But the facts that the mixed infection of the flora resistant against the broad spectrum antibiotics with candida can kill the mice very early and in a high rate, should be suggestive of the possibility of the new mechanism concerning to the development of moniliasis.

Discussion

It is obviously recognized that the incidence of moniliasis in this country has not been so frequent, as described above. However, it is supposed that the recent incline of the practical use of antibiotics might result in the spreading of this disease. Since the prognosis of moniliasis patients is known to be very serious, the study for adequate treatment and prophylaxis against this illness has been essentially requested by those who are dealing with mycotic infections.

In connection with this, researches for the factor, which determine the occurrence of moniliasis should be the basis. The authors consider that the following are some results obtained from our experiments which deserve attention of the researchers.

1) *Candida albicans* is the saprophytes of healthy persons. In the tuberculous patients the incidence of these organisms is especially more frequent. The common locus of the saprophytes is the oral cavity, especially on the furred tongue or in carious teeth. It must be recognized that the desinfection of the oral cavity may be the first step for the prophylaxis of moniliasis. The authors have proven that to deserve to try washing of the oral cavity with the solution of merthiolate from our data for the experimental studies for therapy.

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2) It is not denial for us to believe that the administration of the antibiotics can promote the incidence of moniliasis. However the mechanism of this phenomenon is considered to be very complex. From our experiments, it is plausible that antibiotics can influence the protein-synthesis in the *Candida* body and, the oxidative phosphorylation in the liver and the phagocytosis of the host. As stated in the last experiments, the bacteria that remained unaffected by antibiotic therapy may promote the pathogenicity of *Candida albicans*, or *vice versa*. So it is necessary to keep remember that the emergence of the development of moniliasis may be caused from long term-chemotherapy.

3) Another expeiment of the authors showed, that the mice or volunteers feeded with *Candida albicans* suspension excreted these organisms during shorter period in the feces after the administration of the anti-candidatic drugs than the controls without the latter. So we can suppose the some prophylatic effects of the combined use of the anti-candidatic drugs in the course of the antibiotic therapy against the incidence of moniliasis.

Summary

The authors report in this paper[1] the summarized clinical data of moniliasis in the realm of internal medicine experienced in Japan[2], the experiments performed in regards to the development of this illness, especially in relation to antibiotic therapy, it was proven to be certain that broad spectrum antibiotics affects enhancing the growth of *Candida* body and injuriously to the host, and the pathogenicity of the disease also increases by mixed infection with *Candida albicans* and *Proteus vulgaris* or *Pseudomonas aeruginosa* which are antibiotic-resistant.

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TREATMENT OF TSUTSUGAMUSHI DISEASE WITH TETRACYCLINE*

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Antibiotic treatment of tsutsugamushi disease was begun with the use of chloramphenicol by SMADEL and his co-workers in patients with this disease in Malaya. The results obtained by them were more brilliant than by chemotherapy initiated by TIERNEY, *et al.* with PABA. The authors have already published reports on the studies of this disease, especially on its antibiotic treatment. The authors now present this paper describing the application of tetracycline (Achromycin) to 9 spontaneous cases and 2 inoculated cases af this disease. The spontaneous cases had contracted the disease at infected areas of the basins of the Agano river and the Shinano river of Niigata Prefecture. Niigata strain of *Rickettsia tsutsugamushi* was used in the inoculated cases used for pyretotherapy (isolated from a spontaneous case that contracted the disease at the Agano river basin).

Our diagnosis and observation of the course of tsutsugamushi disease are usually based on leucopenia, demonstration of rickettsia in the blood, WEIL-FELIX reaction, OHTANI's phagocytosis for *Proteus* OXK, *etc.* Diagnosis and seasonal factors, ulcers and scabs due to bites, enlargement of the regional lymph nodes, appearance of rash, *etc.*

Case 1. T. H., a 57-year-old woman (farmeress)

Patient was engaged in agriculture from the latter part of June, 1954, at the tsutsugamushi infected basin of the Agano river. The patient was bitten on an unidentified day, and in the afternoon of August 11 developed fever with chills and shudder. At the same time, her right axillary lymph node was swollen and painful. The patient found a bite on the right posterior axillar line on the level of the 5th rib. For six and a half days following the onset of the disease, *i. e.* August 12th to the 18th forenoon, the paitent was given tetracycline, 50 mg twice daily or 100 mg daily. For 4 days from the afternoon of 18th to the forenoon of 22nd, the patient received 100 mg twice a day; and for 3 days from 22nd afternoon to 25th forenoon, the dosage was modified to 50 mg twice daily. The dosage was further reduced for the period August 26th to 31st, to 25 mg once a day. However, since the patient complained of a sense of fullness in the head and absence of appetite, from 31st for a day and a half, she was given 100 mg twice daily, then for 7 days 50 mg twice daily, the total for the 19 days being 3 g. Still the patient was not relieved of languor. Fever returned on the 11th day after termination of administration. From the 5th day of the returned fever, tetracycline administration was resumed, 100 mg twice daily for 4 days, to a total of 800 mg. This brought the temperature again to normal. Twenty-four days' observations thereafter noted no rise in temperature again. The total amount of tetracycline given was 3.8 g.

Case 2. K. K., a 35-year-old man (farmer)

From the latter part of June to August 10th, 1954, the patient was daily mowing at the basin of the Agano river. He did not know he was bitten, but on August 16 had a sense of heat and vomitted. Three days later, the patient found a rash. He was first examined on August 22nd, 7 days after the onset of the disease. From 22nd until 25th forenoon, *i. e.* for 3.5 days, he was given 50 mg tetracycline twice daily. In the afternoon of the 25th, he received 100 mg, and on the next day 100 mg twice. For 3 days from 27th to 29th, the patient was given 50 mg twice daily; for 5 days from 30th to September 3rd, again 100 mg twice daily; for 5 days from 4th to 8th, 50 mg twice a day; and on both 9th and 10th, 100 mg twice daily. The total amount given over the period of 20 days was 2.8 g. Subsequent 21 days' observations found no relapse.

Case 3. T. I., a 35-year-old woman (farmeress)

The patient was engaged in farm work at the basin of the Agano river from August 10th to 13th. Without being aware of a mite's bite, on August 18th the patient had headache, sense of heat, and generalized languor. When first seen on August 20th, the patient was found to have a bite on the left neck. The lymph node at the left neck was the size of a little finger tip, and the lymph node below

* The trade name for tetracycline is Achromycin.

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the left lower jaw was as large as a thumb tip. Both lymph nodes were tender. Tetracycline 50 mg was given twice daily for 11 consecutive days from August 20th to 30th; and a single daily dose of 25 mg for 8 days from August 31th, making a total of 1.5 g. Following the institution of treatment, the temperature gradually dropped, until it was lower than 37°C on the 11th day of medication. The appetite grew better and subjective symptoms completely relieved from the 4th day of administration. Observation for 2 weeks after the termination of administration found no relapse.

Case 4. Y. O., a 54-year-old man (farmer):

The patient was daily in farm work at the basin of the Agano river from about June, 1954. The patient did not know when he was bitten, but on August 24th developed a fever, with generalized languor, headache and loss of appetite. A bite was noticed on the outer side of the left forearm: at the left armpit were on palpatation two lymph nodes the size of a thumb tip and a pea, respectively, were found, both being painful on pressure. Administration of tetracycline 50 mg was begun on the next day, August 25th, twice daily; and for 3 days from 26th to 28th, 100 mg twice a day; for 3 days from 29th to 31st, 50 mg twice daily; on September 1st and 2nd 100 mg twice a day; for 6 days from 3rd to 8th, 50 mg twice a day. The total for 15 days was 2.0 g. In 5 days after the institution of tetracycline treatment, there were no subjective symptoms at all; and from 10th day, the lymph nodes were no longer tender. Two weeks' observations after termination of medication found no relapse.

Case 5. T. T., a 77-year-old man (farmer)

During his work at the basin of the Agano river on July 1st, 1954, the patient knew he was bitten. The next day he corroded the bite with carbolic acid. From about 16th the same month, the patient had a sense of heat, loss of appetite, headache, and lumbago. On his first examination on 18th, the bite was noticed at the right side of the abdomen. Tenderness was present at the right inguinal lymphatic gland, and there was a rash The administration of tetracycline was started at once with 100 mg twice daily on 18th and 19th. The dosage for 5 days from July 20th to 24th was 50 mg twice a day, on 25th 100 mg twice, and for 4 days from 26th to 29th 50 mg twice a day again. The total amount thus given over the period of 12 days was 1.5 g. The subjective symptoms were relieved on the 11th day of medication. In 8 days after the termination of treatment, the patient was feverish again This fever was brought under control with 50 mg daily for 2 days. The total amount given was 1.7 g. During 3 weeks' observations after that, no relapse was noted.

Case 6. M. I., a 40-year-old man (farmer)

From about the end of June, 1954, the patient was daily at work at the basin of the Agano river. The patient did not know he was bitten, and had on July 12th chills and malaise. Finding a bite each at the right abdomen and scrotum, he corroded the bites with carbolic acid. When first seen on July 14th, the patients right inguinal lymph node had been swollen, tenderness and rash was demonstrated. The patient was immediately put on tetracycline, beginning with 100 mg twice daily for 2 days, followed by 50 mg twice daily for 11 days until 26th. A total of 1.5 g was used over the period of 13 days. On the 12th day after termination of tetracycline administration, there was relapse, with a fever at 37.8°C. From the 4th day of relapse, tetracycline 50 mg was resumed, daily for 2 days, which lowered the temperature to normal. Sixteen-days observation after the termination of the resumed administration of tetracycline found no rise in temperature again. For the entire course, a total of 1.7 g was used.

Case 7. K. U., a 21-year-old man (farmer)

At the Agano river basin on September 3rd, 1954, the patient was bitten one area each at the left and right armpit, the traces of bite were cut away promptly. On September 11th, the patient had a sense of heat and generalized languor. When initially examined on 12th, he was found to have a rash. Tetracycline treatment was then started, with 50 mg twice daily for 6.5 days from 12th afternoon to 18th. The subjective symptoms were removed on 18th. For 17 days from September 19th to October 5th, 25 mg twice daily was given. Th etotal of tetracycline so administered in the 24 days was 1.5 g. Observation was kept on for 28 days after treatment ended, and no relapse was observed.

Case 8. K S., a 44-year-old man (farmer)

Engaing in agricultural operations at the Agano river basin, on August 1st the patient felt a pain in the right ear, without knowing he was bitten by a mite On August 10th, he had a sense of heat. When first examined on August 13th, the patient had at race of bite at the right external auditory canal, with the lymph nodes of the right neck and occipital region enlarged each to the size of a thumb tip and with much tenderness. The spleen was palpated for half a finger's breadth, and the leucocyte count was 4,000 per ml. On August 13th (the 4th day from the onset of disease), the patient was given

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tetracycline, 100 mg in the forenoon and 50 mg in the afternoon. For the subsequent 9.5 days from 14th to 23rd a. m., 50 mg was administered twice daily, followed by 25 mg twice daily for 3.5 days from the 23rd p. m. to 26th, and then 25 mg once daily for 5 days from 27th to 31st. A total of 1.4 g tetracycline was given over the period of 19 days. On the 10th day of treatment, the temperature was lower than 37°C., and no relapse has since been noted.

Case 9. K. S., a 56-year-old man (farmer)

The patient was mowing at the basin of the Agano river on July 6, 1954 when he had no consciousness of being bitten. But, on July 14th, the patient had chills and a fever of 37.5°C. When first examined on July 16th, the 3rd day from the onset of disease, histemperature was 38.6°C., with a trace of bite noticeable on the left side of the scrotum. The bilateral inguinal lymph nodes were enlarged the size of a little finger tip to a thumb tip, and were tender. He had headache and dizziness, but no rash. Tetracycline 100 mg was administered twice daily for 2 days on 16th and 17th, followed by 50 mg twice daily for 11 days from 18th to 28th. The 13 days' total was 1.5 g. The temperature was normal and the headache and dizziness were relieved on the 5th day after the institution of treatment. After termination of medication, the patient rode bicycle and drank a little alcoholic beverage. On the 10th day after treatment ended, the patient had a sense of heat, headache, and maladise, with a fever 38.7°C. The luecocyte count was 5,200 per ml, and rickettsia was demonstrated in the blood. The administration of tetracycline was promptly resumed, 50 mg twice daily for 2.5 days. This total of 250 mg lowered the temperature to normal. A slight fever returned after 3 days of normal temperature. For 2 more days, tetracycline was given 50 mg twice daily (a total of 200 mg). After that the patient was engaged in the usual work, and no relapse was seen. The grand total given was 1.95 g.

Case 10. T. K., a 25-year-old man

This was a patient with schizophrenia in the Niigata Prefectural Mental Institution. The patient intracutaneously inoculated on the inner side of the thigh with 0.5 cc of a 1:10 emulsion of the liver of a mouse that had a Niigata strain of *Rickettsia tsutsugamushi* isolated from a human patient who contracted the disease at the Agano river basin. The disease developed on the 9th day after inoculation. In the afternoon of the 3rd day of development of the disease, he was given 50 mg of tetracycline. For 5 days from the next day, 50 mg was given twice daily followed by 5 more days' 50 mg once a day. For the 11 days, a total of 0.8 g was administered. The temperature was lowered to normal on the 10th day after institution of treatment. No relapse occurred.

Case 11. S. S. a 27-year-old woman

Inoculation was performed as in Case 10. The disease developed on the 11th day thereafter. In the afternoon of the 2nd day of development of the disease, she was given 50 mg tetracycline and for 5 days from the next day 50 mg twice daily, followed by another 50 mg once a day for 5 days. The total for the 11 days was 0.8 g. On the 4th day of administration, the temperature returned to normal. No relapse was noted.

The authors have described the results of tetracycline treatment of 9 spontaneous cases (cases 1 to 9) and 2 inoculated cases (cases 10 and 11) of tsutsugamushi fever. The two inoculated cases were cured with 0.8 g of the antibiotic, and had no relapse. Relapse occurred in the spontaneous cases; Case 1 to which 3 g was given, Case 4 to which 2 g given, and Cases 5, 6 and 9 to which 1.5 g given respectively. All the relapses were cured with less than 1 g of tetracycline, mostly with 0.2 g or so, without another relapse. One case given 2.0 g (Case 4) and another case given 1.5 g (Case 3) showed a slight fever of 37°C for 2 to 4 days and 2 to 3 days, respectively, after termination of medication. These 2 cases, which may be regarded as subrelapse cases regained normal temperature spontaneously without re-medication. Cases 2, 7, and 8 which received 2.8 g, 1.5 g and 1.4 g respectively, were smoothly cured without relapses.

In our previous papers, we have reported that (1) in the treatment of patients with tsutsugamushi fever, if defervescence is rapidly induced by early use of antibiotics, relapse is often unavoidable; and (2) in order to prevent relapses, massive dosages of antibiotics are necessary (at least 3.5 g of chlortetracycline or at least 8 g of chloramphenicol). The authors have further described that if an antibiotic is given after the fever is allowed to continue for about a week after onset, a comparatively small dosage (1.5 g of chlortetracycline, for instance) can cure the disease without relapse.

Our studies further conducted have found a dosage pattern that can be started right at the onset of the disease. This is a slow and gentle manner of administration, and yet is almost sure to cure tsutsugamushi disease without relapse. It consists in the use of 1.5 g of chlortetracycline distributing it over a period of 3 weeks or so. For instance, until the temperature has returned to normal, chlortetracycline is given 50 mg twice daily, and after defervescence 25 mg twice a day.

The effects of various antibiotics on tsutsugamushi disease have been comparatively studied by the authors judging the effects by the days of survival of the mice inoculated with *Rickettsia tsutsugamushi*.

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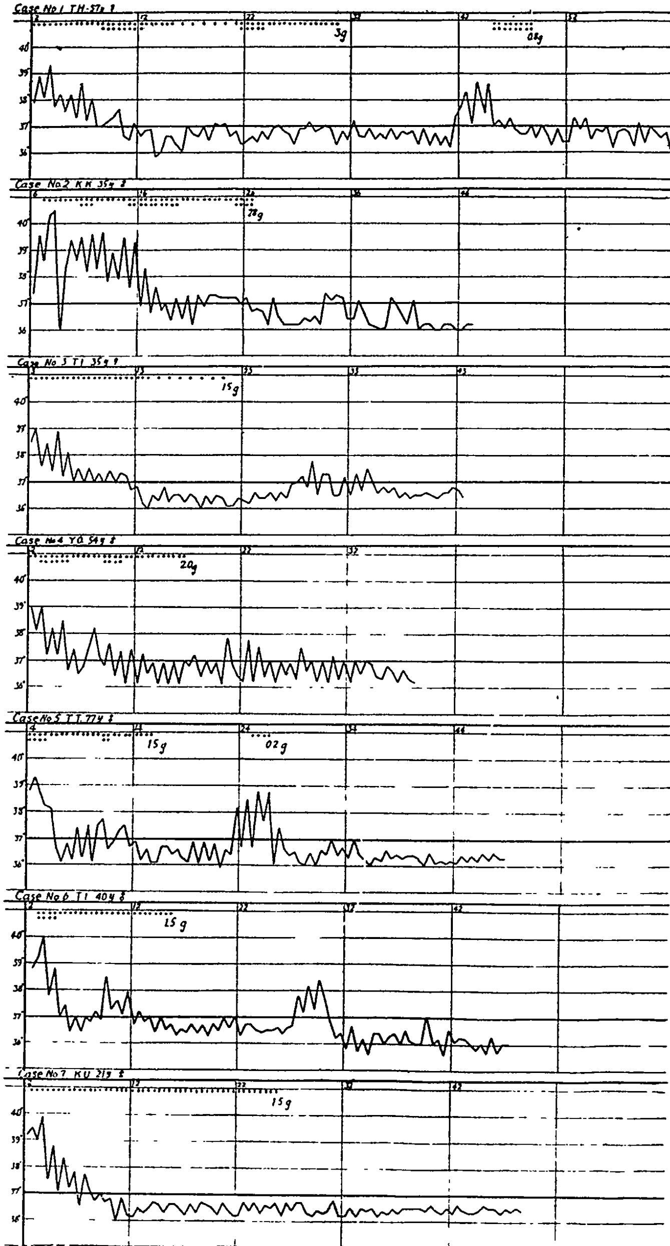

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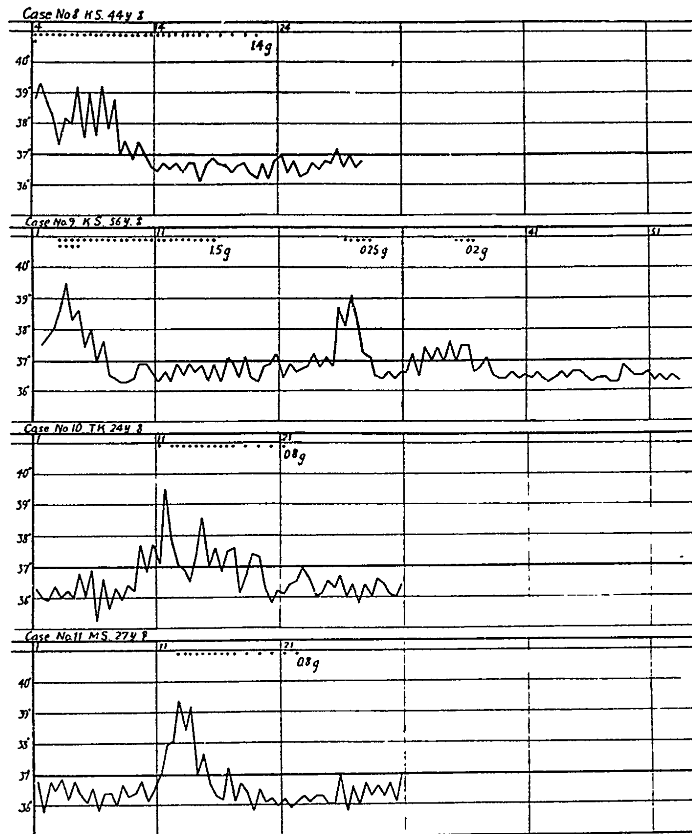

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Tetracycline has been found a little less effective than chlortetracycline. Difference between these two antibiotics when used in human clinical cases has been small, but similar to what has been experienced with the inoculated mice. As compared with chloramphenicol, erythromycin, carbomycin, and leucomycin, tetracycline is a fairly more powerful agent for the treatment of tsutsugamushi fever.

In the antibiotic therapy of this disease, the duration of administration of the drug is as important as the time to begin its use. In all the cases in this report, with the exception of Case 1, we have found that the longer the duration of administration, the less is the relapse is liable to occur even on the same dosage.

From the above results with tetracycline and our experiences with chlortetracycline, the authors conclude that tetracycline is best used against tsutsugamushi fever in the following way:

Until the temperature is lower than 37.2°C for a whole day, tetracycline 100 mg is given twice daily; after defervescence, 50 mg twice a day. This mode of administration is continued for a period of 3 weeks after its institution. The entire amount of tetracycline so given is 2.0 to 2.5 g. This way of medication is believed to be able to cure tsutsugamushi fever smoothly and without relapse.

Apart from its comparison with chlortetracycline, tetracycline is considered a very valuable antibiotic for the treatment of tsutsugamushi disease.

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STUDIES ON CARZINOPHILIN. I

THE PROPERTIES OF CARZINOPHILIN A

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(Received for publication August 3, 1955)

In 1954, it was found by Dr. HATA and his co-workers[1),2)] that an antibiotic obtained from culture broth of a new strain of streptomyces, exhibits inhibitory activity to the growth of YOSHIDA sarcoma. Dr. SHIMADA and his co-workers[3)] reported the promising results on clinical applications of the antibiotic in the field of cancer chemotherapy. The purification study in our laboratory has now resulted in the isolation of an active fraction of carzinophilin in crystalline state after successive treatment of crude powder with organic solvents. The active fraction is designated "carzinophilin A" in this report.

Experimentals

Chemical Properties of Carzinophilin A

The colorless needle-like crystal obtained by repeated recrystallization from organic solvent solution gives the following properties.

Reactions: The antibiotic contains acidic groups. The alkaline and alkaline earth salts can be easily prepared from this free type crystal.

Chief positive reactions:

BAYER's reaction ($KMnO_4$ in NaOH)	+ (green)	Sodium 1, 2-naphthoquinone-4-sulfonate	+ (yellow)
Xanthoprotein	+	Sodium nitroprusside (for active methylene or amino radical)	+
Ninhydrin	+ (yellow)		
Bromine absorption	+	Diphenylamine (for nucleic acid)	+ (deep green)
2,4-Dinitrophenylhydrazine	+		+ (yellow)
Neubauer-Rhode (on heating)	+	Anthrone	

Solubility: Soluble in acetone, chloroform, ethyl acetate, butyl acetate, benzene, dioxane and dilute alkaline water; slightly soluble or insoluble in methanol, ethanol, ether, carbon tetrachloride, petroleum ether and water.

Decomposition products: Following two crystalline substances, N-78 and N-177, were obtained by alkaline decomposition.

N-78: m. p. 78~79°C, colorless needle crystal. C 73.03%, H 6.22%.
Molecular weight: 235 (RAST's method). $C_{14}H_{13\sim14}O_3$

N-177: m. p. 177~180°C, colorless granular crystal. C 71.31%, H 5.68%.
Molecular weight: 214 (RAST's method). $C_{12\sim13}H_{12\sim13}O_3$

Physical Properties of Carzinophilin A

Melting point: 217~222°C (decomp.), after gradual darkening over 205°C.

Constitution: C 59.5~60.0%, H 4.94~5.40%, N 6.76~7.09%; sulfur and halogens are not detected.

Molecular weight:
about 900~1,200 (by RAST's method in bromoform)
about 800~1,200 (by electroconductmetry)

Ultraviolet absorption spectrum: Depending on the kinds of solvents used, the ultraviolet absorption spectrum varies.

Solvents	Absorption maxima (mμ)	Solvents	Absorption maxima (mμ)
2% $NaHCO_3$	218, 250, 283	Chloroform	270
N/10 NaOH	230 ($E^{1\%}_{1cm} \fallingdotseq 940$), 283 ($E^{1\%}_{1cm} \fallingdotseq 460$)	Dioxane	250
N/10 HCl	216, 250	Benzene	282
Methanol	218, 250, 283	Ethyl acetate	255
Ethanol	219, 251, 290	Carbon tetrachloride	262
Ether	219, 252	Carbon disulfide	242, 378
Acetone	210, 330	Ethylene dichlcride	255

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Infrared absorption spectrum: It exhibits characteristic absorption in the infrared region with following maxima: (in chloroform solution).

2.78μ, 2.90μ, 3.03μ, 3.30~3.45μ, 5.80~5.85μ (triplet), 6.10~6.26μ (triplet), 6.45μ, 6.68μ, 6.85μ, 7.10μ, 7.20μ, 7.33μ, 7.50~7.60μ, 7.82μ, 7.95~8.20μ (triplet), 8.47μ, 8.58μ, 8.89μ, 9.13~9.30μ (triplet), 9.35μ, 9.60μ, 10.44μ, 10.80μ, 11.10μ, 11.30μ, 11.75μ, 12.40μ, 13.2μ, 13.5μ,

Polarization: $[\alpha]^{28^\circ}_{D} = +57.8^\circ$ (in chloroform)

Antitumor Activity against YOSHIDA Sarcoma

Each rat was inoculated with 0.2 ml of cell suspension of YOSHIDA sarcoma in physiological saline solution (10 million cells). Twenty-four hours after the inoculation, a single injection of each concentration of carzinophilin A was given intraperitoneally. When a dose of 3 mcg/kg was injected, the sarcoma cells decreased remarkably and did not multiply again during 5 days. With dosis of 1~2 mcg/kg, the number of sarcoma cells decreased immediately, but within 2 or 3 days the number of cells increased gradually. From these data, it will be seen that the antitumor activity of carzinophilin A crystal has been enhanced about 20 times as compared with that of first prepared crude carzinophilin powder[1)2)].

Acute Toxicity

LD_{50} (intravenously to mice) is 150 mcg/kg and LD_0 is probably about 25~50 mcg/kg.

Stability

In dry state, crystalline carzinophilin A is fairly stable. For example, heating at 60°C for 3 hours causes no decrease in activity. Heating at 100°C for 7 hours results in only 10% destruction, and for 20 hours in 30% destruction of original potency. But when exposed to humid atmosphere, the stability is easily lowered and potency is lost rapidly.

In the state of aqueous solution, stability is very low, and cannot be stored for a long time especially in alkaline or acidic solution.

Potency of carzinophilin solution is rapidly destroyed by the addition of the following substances: Thiourea, thioglycolic acid, cystein, thioglycerin, methionine, hydroquinone, anthraquinon, vitamin B2, chlorophyllin, hydrogene peroxide, and formalin.

The activity of aqueous solution is also destroyed by the radiation of ultraviolet ray.

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ON THE CLASSIFICATION OF ANTIFUNGAL ANTIBIOTICS

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In recent years, a number of antifungal substances produced by streptomyces, fungi and bacteria have been isolated. In our laboratory also, efforts have been made to find out antifungal antibiotics produced by streptomyces, and some new substances were isolated[12,16,24,26].

In order to differenciate new antifungal antibiotics from known substances rapidly, some simple methods are required. Several antifungal antibiotics produced by streptomyces have been classified into 4 groups by TAKAHASHI[1] in our laboratory by the application of ISHIDA's summarized papergram[2] which had been developed for the identification of antibacterial antibiotics.

The present paper deals with the classification of antifungal antibiotics by means of summarized papergram, and comparative studies of the above classification with those results obtained by the electrophoretic papergram[3], diffusion curve, antifungal spectrum, and the identification by the ultraviolet absorption spectrum emphasized by UMEZAWA and others[4].

Meterials and Methods

1. Antifungal antibiotics: Following 24 antibiotics were tested.

Antibiotics	Species	Supplier
Actidione[5]	*Str. griseus*	EVANS, J. S.; Upjohn Co.
Ascosin[6]	*Str. canescus*	HIDY, P. H.; Commercial Solvent Corp.
Aureothricin[7]	*Str. thioluteus*	UMEZAWA, H.; National Inst. of Health of Japan
Bacillomycin[8]	*Bac. subtilis*	WARREN, G. H.; Weyth Inst.
Candicidin[9]	*Str. griseolus*	WAKSMAN, S. A.; Rutgers Univ.
Candidulin[10]	*Asp. candidus*	STANSLY, P. G.; Detroit Inst. of Cancer Research
Clavacin[11]	*Asp. clavatus*	WAKSMAN, S. A.; Rutgers Univ.
Datemycin[12]	*Bac. subtilis*	Dept. Bacteriol., Tohoku Univ.
Endomycin[13]	*Str. albus*	GOTTLIEB, D.; Univ. of Illinois
Eurocidin[14]	*Str. eurocidicus*	NAKAZAWA, K.; Inst. for Fermentation, Takeda
Fermicidin[15]	*Str. griseolus*	NAKAZAWA, K.; Inst. for Fermentation, Takeda
Flavacid[16]	*Str.* No. 0-2 strain	Dept. Bacteriol., Tohoku Univ.
Fungicidin[17]	*Str. norsei*	REED, G. S.; Squibb Inst.
Griseofulvin[18]	*Pen. griseofulvum*	BRIAN, P. W.; Butterwick Res. Lab.
Hygroscopin A[19]	*Str. hygroscopicus*	NAKAZAWA, K.; Inst. for Fermentation, Takeda
Mycelin[20]	*Str. roseoflavus*	AISO, W.; Inst. for Putrefaction Res., Chiba Univ.
Mycosubtilin[21]	*Bac. subtilis*	WALTON, R. B.; Merck & Co., Inc.
Rimocidin[22]	*Str. rimosus*	TANNER, F. W.; Chas. Pfizer & Co.
Thiolutin[23]	*Str. albus*	TANNER, F. W.; Chas. Pfizer & Co.
Toyokamycin[24]	*Str. albus*	Dept. Bacteriol., Tohoku Univ.
Trichomycin[25]	*Str. hachijoensis*	HOSOYA, S.; Inst. for Inf. Dis., Tokyo Univ.
Trichonin[26]	*Str. rubrireticuli*	Dept. Bacteriol., Tohoku Univ.
Viridin[27]	*Trichoderma viride*	BRIAN, P. W.; Butterwick Res. Lab.
E-150 Substance[28]	*Str.* No. E-150 Strain	Dept. Bacteriol., Tohoku Univ.

2. Summarized papergram:

(a) The experiments were performed principally by the method reported by ISHIDA and others[2].

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Solvents used were as follows:

A. Wet n-butanol B. 20% NH_4Cl
C. 75% Phenol D. 50% Acetone
E. 40 cc Butanol+10 cc methanol+20 cc aq. dest.+1.5 g methylorange
F. 40 cc Butanol+10 cc methanol+20 cc aq. dest.
G. 80% Benzole+20% methanol H. Aq. dest.

(b) Solvents used for dissolving the samples were as follows:

1) Water: Actidione, fermicidin, clavacin, candicidin, datemycin, toyokamycin, and E-150 substance.
2) Chloroform: Griseofulvin, and viridin.
3) Other antibiotics.

3. Electrophoretic papergram: The experiments were routinely performed by TAKAHASHI's method[5], under the condition of 200 volt for 150 minutes at pH 5.0 and 8.0 of 1/15 M phosphate buffers.

4. Diffusion curve: Pulp-disc method was employed in assaying antibiotic potency. Test organisms used were either *Candida albicans* (M-9) or *Saccharomyces cerevisiae*, and the solvents used for dissolving the materials were the same as in summarized papergram.

5. Ultraviolet absorption spectrum: Beckman's electrophotometer was used. Datemycin, E-150 Substance, and toyokamycin were dissolved in water, griseofulvin and viridin in chloroform, and others in methanol. The concentration of each material was 10 mcg per ml, except 100 mcg per ml of actidione.

6. Antifungal spectrum: The experiment was carried out by agar dilution streak method. Eleven species of test organisms were streaked on 1% glucose nutrient agar containing serially diluted concentrations of 100 mcg to 0.1 mcg per ml of each sample, and they were incubated at 27°C for 24 or 48 hours.

Experimental Results

Twenty-four antibiotics were classified into 11 groups by summarized papergram.

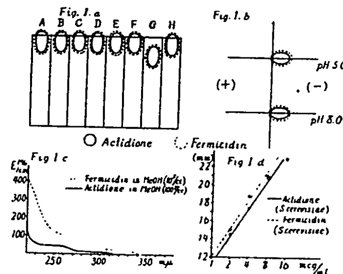
I. ANTIFUNGAL ANTIBIOTICS PRODUCED BY STREPTOMYCES

I Group: Actidione, and fermicidin

This group showed higher Rf-values with every kind of solvents (Fig. 1. a). Both antibiotics exhibited the similar characteristics in electrophoretic papergram, ultraviolet absorption, diffusion curve (Fig. 1. b~d), and antifungal spectrum (Table 1).

II Group: Hygroscopin A, trichonin, mycelin, and ascosin (1 st substance)*

The antibiotics in this group migrated only slightly or not at all with B solvent, but to the top with other solvents. In this group mycelin and ascosin (1 st substance) showed lower Rf-values with H solvent (Fig. 2. a). The electrophoretic papergram indicated that trichonin has a tendency to migrate towards the cathode, while others did not migrate from the original spots (Fig. 2. b). In ultraviolet absorption spectrum, diffusion curve (Fig. 2. c~d), and antifungal spectrum (Table 1), all substances were different from each other.



III Group: Toyokamycin

This group showed middle Rf-values with all the solvents employed except G solvent (Fig. 3. a). Toyokamycin alone belonged to this group. It exhibited a slightly basic charge in electrophoretic papergram, a characteristic ultraviolet absorption, and diffusion curve (Fig. 3. b~d).

IV Group: Aureothricin, and thiolutin

This group showed lower Rf-values with B and H solvents, higher Rf-value with C solvent, and middle Rf-values with other solvents. In the solubilities to various kinds of solvents, this group resembled to III Group. It was already reported that aureothricin and thiolutin are closely related in their physicochemical natures, and they bore remarkable resemblance in electrophoretic papergram and ultraviolet absorption (Fig. 4. b~c). The diffusion curve of these substances could not be obtained by the pulp disc method, because of their very low diffusibility.

* The sample of ascosin used in this experiments contained two antifungal substances. The 1 st substance had a weak activity, and the 2 nd substance stronger activity. The 2 nd substance belonged to VI Group.

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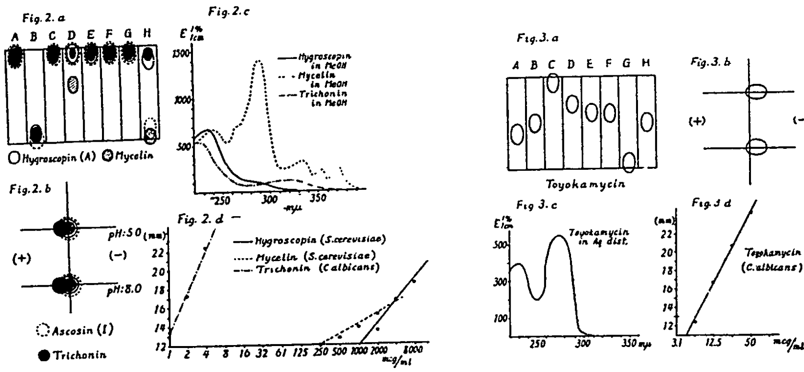
Table 1. Antifungal Spectrum

Group	Produced by Streptomyces	Test organisms										
		C. albicans (Takeda)*	*C. krusei**	*T. interdigitale***	*Willia**	*Torula utilis**	*S. cerevisiae***	*Botritis***	*Absidia orchidis**	*Mucor pusilis***	*Asp. niger***	*B. subtilis* (558)*
1	Actidione	100	0.4	100	0.2	0.4	0.2	1.6	1.6	6.2	3.1	>100
	Fermicidin	50	0.4	100	0.1	0.2	0.2	0.8	3.1	25	1.6	100
2	Hygroscopin	100	>100	100	100	100	100	100	25	100	100	100
	Trichonin	100	6.3	100	3.2	1.6	6.3	3.2	50	50	12.5	100
3	Toyokamycin	1.6	>100	>100	100	>100	6.2		>100	>100	>100	>100
4	Aureothricin	3.1	50	50	12.5	3.1	12.5	25	12.5	1.6	25	12.5
	Thiolutin	6.2	100	50	12.5	1.6	25	25	12.5	3.1	25	12.5
5	Fungicidin	1.6	6.2	6.2	3.1	3.1	0.8	1.6	3.1	1.6	6.2	>100
	Rimocidin	1.6	3.1	3.1	1.6	1.6	1.6	1.6	0.8	1.6	1.6	>100
	Candicidin	1.6	1.6	100	3.1	1.6	6.2	6.2	50	25	12.5	100
	Flavacid	25	12.5	100	25	25	3.1	12.5	50	50	25	12.5
	Endomycin	6.2	25	100	25	12.5	50	12.5		100	12.5	>100
6	Trichomycin	<0.1	<0.1	>100	0.2	<0.1	<0.1	0.4	12.5	1.6	0.4	50
	Ascosin	0.2	0.4	25	0.8	0.4	1.6	6.2	6.2	50	1.6	100
	Eurocidin	1.6	3.1	12.5	3.1	1.6	1.6	1.6	0.4	6.2	1.6	100
7	E 150 substance	6.3	>100	12.5	>100	100	>100			100	100	>100

* Incubated for 24 hrs. ** Incubated for 48 hrs.



V Group: Fungicidin, rimocidin, candicidin, flavacid, and endomycin

The antibiotics belonging to this group remained at the original spots or only slightly migrated with B, G and H solvents, except endomycin which showed a higher Rf-value with H solvent (Fig. 5. a). The patterns of this group bore some resemblance to that of VI Group. Amon, them, fungicidin and rimocidin exhibited similar characteristics in summarized papergram, electrophoretic papergram, and ultraviolet absorption (Fig. 5. b~c), though their diffusion curves revealed a slight difference from each other

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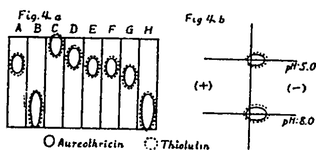
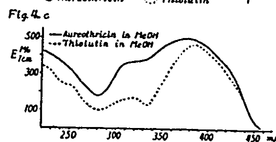
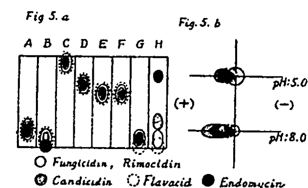
(Fig. 5. d). Candicidin, flavacid and endomycin differed from the formers in electrophoretic papergram. These 3 substances migrated towards the cathode, while the formers remained at original spots. The latter 3 substances differed from each other and from the formers in summarized papergram with H solvent, ultraviolet absorption, diffusion curve, and antifungal spectrum. It is noteworthy that the summarized papergram of candicidin differed from those of trichomycin (VI Group) and ascosin 2 nd substance (VI Group), while the ultraviolet absorption spectra of these substances resembled very much with each other.

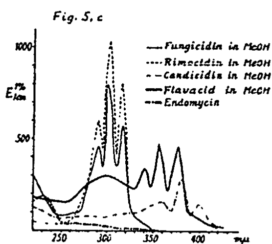
VI Group: Trichomycin, ascosin (2 nd substance), and eurocidin

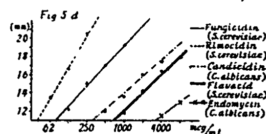
This group showed no movement with A, B, D, G

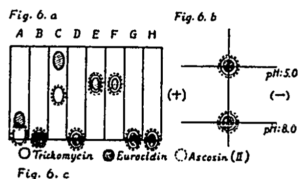


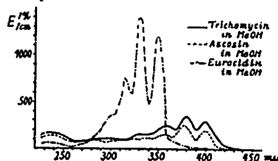


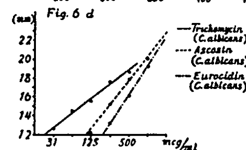












and H solvents, except eurocidin showed a slight migration with A solvent, and a higher Rf-value with C solvent (Fig. 6. a). Trichomycin and ascosin 2 nd substance gave similar patterns in ultraviolet absorption and electrophoretic papergram (Fig. 6. b~c), but differed in diffusion curve and antifungal spectrum (Fig. 6. d). In the latter points, ascosin (2 nd substance) seemed to be more related to eurocidin than trichomycin. It is very interseting that, except eurocidin, the antibiotics belonging to V and VI Groups are chiefly obtained from mycelium and they have 3 characteristic maxima in ultraviolet absorption spectrum.

VII Group: E-150 Substance

E-150 Substance alone belonged to this group. It migrated to the top with B solvent, remained at the original spots with A, C, and G solvents, and slightly migrated with other solvents (Fig. 7. a). In electrophoretic papergram, it exhibited a good migration towards the anode in alkaline or acidic buffer (Fig. 7. b). Its ultraviolet absorption was also characteristic (Fig. 7. c). Diffusion curve of the antibiotic could not be obtained, because the border of inhibition zone was not clear.

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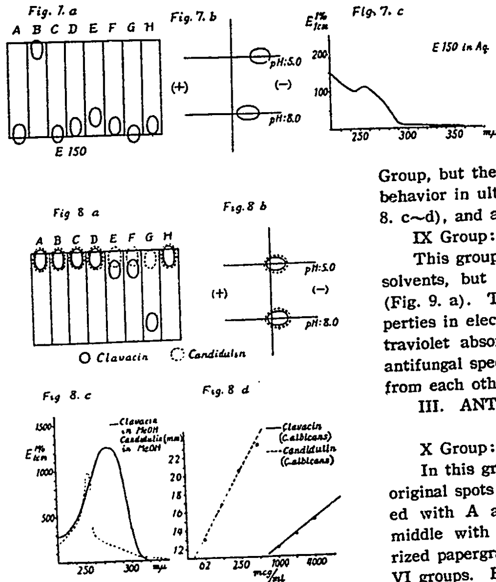

II. ANTIFUNGAL ANTIBIOTICS PRODUCED BY FUNGI

VIII Group: Clavacin, and candidulin

Clavacin and candidulin belonged to this group. The summarized papergram and electrophoretic papergram (Fig. 8. a~b) of this group was greatly related with that of I Group, but these two substances showed quite different behavior in ultraviolet absorption, diffusion curve (Fig. 8. c~d), and antifungal spectrum (Table 2).

IX Group: Viridin and griseofulvin

This group indicated middle Rf-values with B and H solvents, but the good migration with other solvents (Fig. 9. a). These substances showed similar basic properties in electrophoretic papergram (Fig. 9. b). In ultraviolet absorption, diffusion curve (Fig. 9. c~d), and antifungal spectrum (Table 2), both substances differed from each other.

III. ANTIFUNGAL ANTIBIOTICS PRODUCED BY BACTERIA

X Group: Bacillomycin, and mycosubtilin

In this group, the inhibition zones remained at the original spots with B, G and H solvents, slightly migrated with A and D solvents, and migrated to about the middle with other solvents (Fig. 10. a). The summarized papergram of this group resembled to that of V or VI groups. Bacillomycin and mycosubtilin were similar

Table 2. Antifungal spectrum

(The minimum concentration (mcg per ml) of complete growth inhibition).

Group		Substance	Test organisms											
			C. albicans (Takeda)*	*C. krusei**	*T. interdigitale***	*Willia**	*Torula utilis**	*S. cerevisiae***	*Botritis***	*Aksidia orchidis**	*Mucor pusilis**	*Asp. niger***	*B. subtilis* (558)*
Produced by Fungi	8	Clavacin	100	100	100	25	25	3.1	25	50	6.2	50	6.2
		Candidulin	0.8	12.5	50	12.5	6.2	6.2	12.5	6.2	12.5	6.2	6.2
	9	Viridin	6.2	25	6.2	25	6.2	6.2	12.5	50	12.5	12.5	1.6
		Griseofulvin	100	100	100	100	25	25	100	100	100	100	100
Produced by Bacilli	10	Bacillomycin	100	100	100	25	25	12.5	100	25	0.8	50	100
		Mycosubtilin	100	100	100	25	6.2	100		12.5	12.5	6.2	>100
	11	Datemycin	50	>100	100	100	100	100	100	100	100	100	>100

* Incubated for 24 hrs. ** Incubated for 48 hrs.

in ultraviolet absorption (Fig. 10. c), but in electrophoretic papergram the former showed a good migration towards the cathode in contrast to the latter. Moreover, these substances differed in their diffusion curves.

XI Group: Datemycin

Datemycin alone was included in this group. It showed a Rf-value of O with G solvent, migrated

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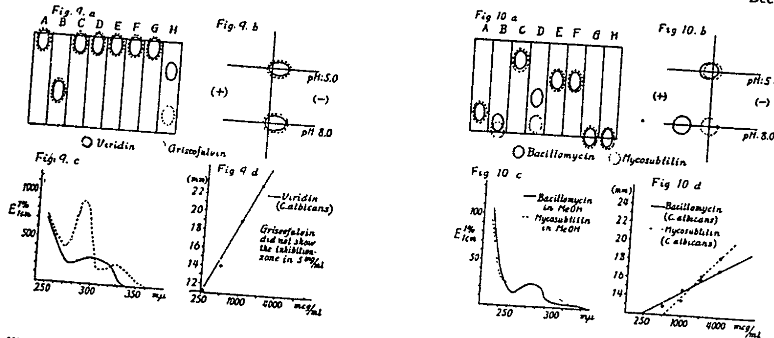
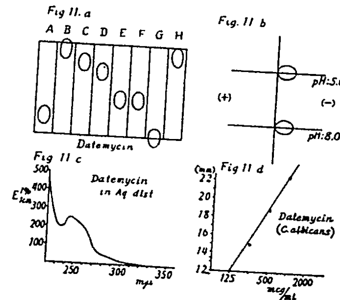


to the upper end with B and H solvents, slightly migrated with A solvent, and showed middle Rf-values with C, D, E and F solvents (Fig. 11. a). In electrophoretic papergram, this substance migrated only slightly towards the anode indicating a basic character (Fig. 11. b). Its ultraviolet absorption spectrum (Fig. 11. c) and antifungal spectrum (Table 2) were also characteristic.



Discussion

In the studies of the screening of antifungal substances, 24 known antifungal antibiotics were classified into 11 groups by the summarized papergram. By the papergrams, we could not differenciate the closely related substances such as actidione and fermicidin. However, the patterns obtained would be useful not only for the identification of antifungal agents produced in agar or broth, but also for the selection of solvents employed in purification procedure. It would be possible to assume that the substances belonging to Groups I, II, VIII and IX may be very soluble in various solvents, and those belonging to Group VII will be scarcely or not soluble in organic solvents.

It is very interesting to observe the relationship between the classifications by summarized papergram and by ultraviolet absorption spectrum. In so-called trichomycin-candicidin-ascosin group classified by ultraviolet absorption spectrum, candicidin belonged to Group V by summarized papergram, while trichomycin and ascosin to Group VI

In the electrophoresis, diffusion curve and antifungal spectrum, such correlations as mentioned above could not be recongnized.

Conclusion

By summarized papergram, the author could classify 17 antifungal substances produced by streptomyces into 7 groups, 4 substances produced by fungi into 2 groups, and 3 substances produced by bacteria into 2 groups.

The author also examines the diffusion curve, antifungal spectrum, electrophoretic papergram and ultraviolet absorption spectrum of each substance, but the results thus obtained gave no close correlation with our classification.

The author wishes to express his sincere thanks to the research workers for the valuable samples, and to Prof. M. KUROYA for his cordial guidance throughout the work.

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THE MECHANISM OF ACTION OF PARA-AMINOSALICYLIC ACID IN PREVENTING THE EMERGENCE OF RESISTANCE TO STREPTOMYCIN IN *MYCOBACTERIUM TUBERCULOSIS*

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GRAESSLE and PIETROWSKI (1949) reported on the *in vitro* effect of para-aminosalicylic acid (PAS) in preventing resistance to streptomycin by *Mycobacterium tuberculosis* and thereafter the effect was clinically confirmed by TEMPEL, *et al.* (1951).

The mechanism of action of PAS in preventing the emergence of streptomycin resistance when used in combination was considered by GRAESSLE and PIETROWSKI as to exist the possibility that the streptomycin-resistant mutants were being inhibited by PAS in the combination. On the other hand, the relationship between synergism and drug resistance was indicated by KLEIN and KALTER (1945) in a report on the combined action of penicillin and the sulfonamides and later by KLEIN and KIMMELMAN (1947) in a report on the combined action of streptomycin and the sulfonamides against *Staphylococcus aureus*. They reported that an important factor in the observed synergism was the ability of a small amount of an added drug to prevent the multiplication of few bacteria resistant to the test concentration of penicillin or streptomycin and that the use of lower concentrations of each drug is an important factor in the decreased resistance to the drug.

The activity of PAS against *M. tuberculosis* when combined with streptomycin *in vitro* was reported by VENNESLAND, *et al.* (1948) to be additive. We found that the *in vitro* activity of streptomycin and PAS against *M. tuberculosis* was synergistic when used in combination It appears to be very probable the possibility exists, which has been indicated by KLEIN, *et al.* However, the following two possibilities of the mechanism of action of PAS in preventing the emergence of streptomycin resistance could be considered:

(1) The emergence of streptomycin resistance by *M. tuberculosis* readily occurs, but the emergence of PAS resistance can occur very slowly. Even if a small number of the streptomycin-resistant cells were selected by the action of streptomycin, these resistant cells could be inhibited by PAS, and the smaller the number of the resistant cells, the smaller the amount of PAS required to inhibit the resistant cells would be expected.

(2) The mutation to streptomycin resistance by *M. tuberculosis* is influenced by the presence of PAS.

The former was considered by KLEIN, *et al.* (1945, 1947) and GRAESSLE, *et al.* (1949). The latter seems to have been not considered. It was considered, therefore, advisable to study what effect PAS might have upon the emergence of streptomycin resistance *in vitro* in *M. tuberculosis.*

Materials and Methods

Mycobacterium tuberculosis var. *hominis*, Frankfurt strain, was used as the test organism. The strain had maintained by monthly transfers on T. OGAWA's medium. The medium consisted of KH_2PO_4 1 g, sodium glutaminate 1 g; in 100 ml distilled water, eggs 200 ml, 2% malachite green 6 ml, and glycerol 6 ml. Each tube contained 10 ml of the medium

Dihydrostreptomycin sulfate (Meiji) and sodium para-aminosalicylate (Tanabe) were used in this study. The drugs were dissolved in distilled water, and appropriate dilutions and combinations of dihydrostreptomycin and sodium para-aminosalicylate were made in OGAWA's medium before steriliztaion. The medium was sterilized at 85～88°C for 40 minutes and slants were obtained. The activity of dihydrostreptomycin after the sterilization was regarded as ½ of the activity before the sterilization. This decrease results from adsorption of dihydrostreptomycin into heat-coagulated protein according to M. OGAWA, *et al.* (1954). The activity of sodium para-aminosalicylate was regarded to be not changed by the sterilization. In the following description, the terms of streptomycin and PAS will be used, although dihydrostreptomycin and sodium para-aminosalicylate were used in the study.

Cell suspensions for the inocula for all tests were prepared by shaking the organism with small glass-balls in flask, suspending it in 0.9% saline and making appropriate dilutions. Wet weight of the suspended cells was standardized by the Leitz's ROUY-Photometer using a filter 610 mμ and comparing a given density with the standard curve previously prepared.

Viable count of the inoculum were determined by inoculating appropriate dilutions of the cell

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suspension used for the inocula upon 10 tubes containing OGAWA's medium and observing the viable counts after approximately 3～4 weeks incubation. All samples used were incubated at 37°C throughout the study.

Results

A. The Effect of PAS in Preventing the Multiplication of the Streptomycin-resistant Cells Survived

1. *M. tuberculosis* which had grown on OGAWA's medium for 4 weeks was inoculated to each tube of a series of tubes containing graded concentrations of streptomycin. The inoclum for each tube consisted of 40 mcg in wet weight and this contained 2.5×10^9 viable counts. The tubes were incubated at 37°C for 6 weeks and the growth was observed every week. In tubes containing the drug in concentrations less than 0.63 mcg per ml, the growth occurred as well as did in the tube containing no drug.

2. After 6 weeks incubation the organism grown on the tube at a concentration of 0.63 mcg per ml of the drug was suspended in saline. The suspension was inoculated to each tube of a fresh series of tubes containing graded concentrations of the drug and incubated at 37°C for 6 weeks. Each inoculum used in the transfer (the second inoculation) consisted of 40 mcg in wet weight. The growth observed in tubes containing the drug in concentrations less than 1.25 mcg per ml was quite similar to that in a tube containing no drug.

3. The organism grown on the tube at a concentration of 0.63 mcg per ml of streptomycin in the second series was again suspended in saline. The organism, which had been exposed to a level of concentration of 0.63 mcg per ml of streptomycin for 12 weeks in the total, which could be regarded as not selective, was inoculated to 3 fresh series of tubes as follows: (a) a series of tubes containing graded concentrations of streptomycin alone, (b) a series of tubes containing graded concentrations of streptomycin in combination with PAS at a concentration of 0.05 mcg per ml of medium, (c) a series of tubes containing graded concentrations of streptomycin in combination with PAS at a concentration of 0.1 mcg per ml of medium.

Each tube of the 3 series was inoculated with 40 mcg of the organism and the inoculum to each tube consisted of 6×10^4 viable cells. The results of the experiment are shown in Table 1. As shown in (a) of the table, the orgranism which had grown on the medium containing 0.63 mcg per ml of streptomycin for 12 weeks represented the same growth even on the medium containing 2.5 mcg per ml of streptomycin as on the medium containing no drug. Even in the tube containing 5 mcg of the drug per ml of the medium the growth occurred, although it was less than that of the contrast. The results indicate that the inoculum had contained a considerable number of the cells resistant to low levels of streptomycin.

Table 1. The *in vitro* effect of streptomycin and PAS against *M. tuberculosis* var. *hominis* when used in combination

Streptomycin mcg per ml	(a) Streptomycin alone	(b) Streptomycin plus PAS 0.05 mcg per ml	(c) Streptomycin plus PAS 0.1 mcg per ml
160	–	–	–
80	–	–	–
40	–	–	–
20	–	–	–
10	–	+	–
5	⧺	⧺	–
2.5	⧻	⧻	⧺
1.25	⧻	⧻	⧺
0.63	⧻ *3	⧻	⧻ *3
0	⧻ *1	⧻	⧻

The results presented were observed after 6-week incubation at 37°C. Each tube was inoculated with 6×10^4 viable cells of *M. tuberculosis* var. *hominis*, Frankfurt strain, which had grown on the medium containing 0.63 mcg of streptomycin per ml of medium.
+.........1～10 colonies; ++.........11～100 colonies;
+++.........several hundred colonies; ++++.........membraneous growth.

A similar result was obtained in the series (b), as shown in (b) of the table. This indicates that the addition of 0.05 mcg per ml of PAS has no effect to prevent the development of the streptomycin-resistant cells.

As shown in (c) of the table, however, the growth similar to the contrast occurred only in tubes containing streptomycin of less than 0.63 mcg per ml and the growth was completely inhibited in a tube containing 5 mcg per ml of streptomycin in the presence of 0.1 mcg per ml of PAS.

The concentration of PAS was never inhibitory when used alone. The results shown in Table 1 indicate, therefore, that the growth of the streptomycin-resistant cells has been inhibited in the presence of both streptomycin and PAS,

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although the concentration of PAS used is never inhibitory when used alone.

Since PAS does not inhibit specifically the streptomycin-resistant cells, the mechanism of action of PAS in preventing the streptomycin-resistant cells appears to be as follows:

All bacterial populations show a variation in the susceptibility of their individual cells to a given concentration of streptomycin. At partially inhibitory concentrations of streptomycin, the surviving cells represent a selection of the most resistant cells in the culture. By addition of PAS, the surviving cells are inhibited by a low concentration of PAS. When only a small number of streptomycin-resistant cells are present, low concentrations of PAS would be particularly effective, even if the concentration of PAS could not inhibit the populations.

B. The Effect of PAS in Preventing the Mutation to Streptomycin-resistance in the Presence of Streptomycin

Since GRAESSLE and PIETROWSKI (1949) found that the strain that had been exposed to the action of both drugs retained its sensitivity to streptomycin or to PAS when used singly, it was considered of interest to determine whether the mutation to streptomycin resistance in the presence of streptomycin was influenced by PAS or not. The problem is, therefore, related to the mutagenic effect of streptomycin (AKIBA and YOKOTA, 1952).

M. tuberculosis which had grown on the medium containing 0.63 mcg per ml of streptomycin for 12 weeks was inoculated to the following media: (1) a medium containing no drug; (2) a medium containing 0.63 mcg of streptomycin per ml; and (3) a medium containing both 0.63 mcg of streptomycin and 0.1 mcg of PAS per ml.

The inoculum used contained 6×10^4 viable cells. The media and the inoculum used correspond to those (*1, *2, and *3) in Table 1 respectively.

The tubes inoculated were incubated for 5 weeks and observed every week The growth on these media was almost similar to each other. After 5-week incubation the organism grown on the media was taken and cell suspensions of 6~10 mg per ml were prepared from each medium, and 0.05 ml of each suspension was inoculated to 20 tubes containing 20 mcg of streptomycin per ml of medium. On the other hand, 0.05 ml of 10^{-5} dilution of each suspension was inoculated to 10 tubes containing no drug. After an incubation period for 4 weeks at 37°C, the number of the cells resistant to 20 mcg of streptomycin per 10^5 viable counts were determined. The results of the experiment were shown in Table 2.

As shown in the table, the population which had grown on the medium containing 0.63 mcg of streptomycin per ml of medium (*2) contained a much larger number of the streptomycin-resistant cells than the population which had grown on the medium containing no drug (*1. However, the population which had grown on the medium containing both 0.63 mcg of streptomycin and 0.1 mcg of PAS per ml of medium (*3) contained a significantly smaller number of the resistant cells than that which had grown on the medium containing streptomycin alone.

Table 2. *Mycobacterium tuberculosis* var. *hominis*, Frankfurt strain

Medium, on whih bacterial populations had grown	Number of cells per inoculum	Number of colonies growing on each plate containing 20 mcg of streptomycin per ml of medium	Number of resistant colonies per 10^7 viable cells
(1)	$303\ (\pm86.2)\times10^5$	75.3 ± 18.9	$24.8/10^7$
(2)	$47.6\ (\pm13.6)\times10^5$	92.0 ± 30.0	$193.3/10^7$
(3)	$299\ (\pm88.1)\times10^5$	145.5 ± 40.6	$48.6/10^7$

(1) A medium containing no drug (*1).
(2) A medium containing 0.63 mcg of streptomycin per ml of medium (*2).
(3) A medium containing 0.63 mcg of streptomycin and 0.1 mcg of PAS per ml of medium (*3).

Discussion

Two possibilities were considered as the mechanism of action of PAS in preventing the emergence of streptomycin-resistant cells.

(A) The effect of PAS to prevent the multiplication of the few cells resistant to the test concentration of streptomycin.

This effect was considered by KLEIN and KALTER (1945) and KLEIN and KIMMELMAN (1947) on the combined effect of penicillin and sulfonamides or streptomycin and sulfonamides. The results shown in our experiment (A) indicate that an important factor in preventing the emergence of streptomycin resistance is the effect of PAS to prevent the small number of the streptomycin-resistrant cells survived, and the results indicate also the presence of synergism between streptomycin and a small amount of PAS.

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(B) The effect of PAS to decrease the mutation to streptomycin resistance in *M. tuberculosis* in the presence of streptomycin.

The results shown in Table 2 indicated that the population which had grown on the medium containing 0.63 mcg of streptomycin per ml of medium contained a much larger proportion of the streptomycin-resistant cells than the population which had grown on the medium containing no drug. However, the increase of proportion of the resistant cells per viable cells observed in the former was considerably diminished when the medium contained both 0.63 mcg of streptomycin and 0.1 mcg of PAS per ml of medium ((3) in Table 2). The concentration of both drugs were subinhibitory when used alone and even in combination.

As an interpretation of this phenomenon the following possibilities would be considered: (1) The most streptomycin-resistant cell sappear in the early stage of growth and the growth curve is retarded by the presence of both drugs. (2) The streptomycin-resistant cells are more susceptible to PAS than the parent sensitive cells. (3) PAS prevents the mutation to streptomycin resistance produced in the presence of streptomycin. (4) Although the selection by streptomycin might appear not to occur, it does occur.

Since the concentration of streptomycin used for selection is very low, the number of the selected streptomycin-resistant cells must be considerably large. Therefore, the growth appears to be similar to the contrast. The selected cells resistant to a low level of streptomycin develop a larger number of more highly resistant cells than do the inhibited cells. In this case, one must suppose that the mutation rate to streptomycin resistance of the selected cells is higher than that of the unselected cells.

The action of PAS in decreasing the emergence of streptomycin-resistant cells, therefore, should be the prevention of mutation inducing the streptomycin resistance, since the concentration of streptomycin used for selection is the same in the cases (2) and (3) in Table 2. (The fourth possibility does not require to suppose the mutagenic effect of streptomycin. Since the drugs in these concentrations were not inhibitory to the growth of the population when used alone and even in combination, it has been considered that the decrease of the emergence of the streptomycin-resistant cells does not result from the mechanism (A).)

Among four possibilities here presented, the first possibility seems to be negligible, since retardation of the growth has never been observed among the media tested.

The second possibility is completely excluded, as the streptomycin-resistant strain has been tested for sensitivity to PAS and has been found to be sensitive as well as the streptomycin-sensitive parent strain under the condition using inocula containing appropriately the same viable counts in each other (TSUKAMURA, 1955, unpublished data). Therefore, there remain the third and the fourth possibilities. Both these possibilities contain the effect of PAS to decrease the mutation to streptomycin resistance.

However, the inoculum used by us had grown on the media containing 0.63 mcg of streptomycin per ml for 12 weeks before inoculation. On the other hand, the number of viable counts of the inoculum obtained on the medium containing no drug was 47.6 ± 13.6 and the number of viable counts of it on the medium containing 0.63 mcg of streptomycin per ml was 42.5 ± 11.2. No significant difference between these numbers is present. Therefore, it seems to be very probable that the selection by the streptomycin titer of 0.63 mcg per ml does not occur. The fourth possibility would be, therefore, considered to be not probable. Thus, the third possibility seems to be most probable. This interpretation is related to the mutagenic effect of streptomycin (AKIBA and YOKOTA, 1952).

The results of the experiment (B) would be explained as follows: The proportion of the streptomycin-resistant cells has been increased by the mutagenic effect of streptomycin in the presence of streptomycin at a concentration of 0.63 mcg per ml, and the mutagenic effect of streptomycin has been antagonized by the addition of PAS at a concentration of 0.1 mcg per ml in the combination.

Summary

The mechanism of action of para-aminosalicylic acid (PAS) in preventing the emergence of the streptomycin-resistant cells in *Mycobacterium tuberculosis* var. *hominis* when used in combination with streptomycin was studied in the present paper, and the following conclusions were obtained.

Two mechanisms of the effect of PAS in preventing the emergence of the streptomycin-resistant cells have been indicated.

It has been found that an important factor in the effect of PAS is the ability of a small amount of the added PAS to prevent the multiplication of the few cells resistant to the test concentration of streptomycin, which have survived the selection of streptomycin, and that the other important factor in the effect of PAS when used in combination with streptomycin is the ability of the added PAS to antagonize the mutation to streptomycin resistance in the presence of streptomycin, that is, to antagonize the mutagenic effect of streptomycin.

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STUDIES ON THE PYRIDOMYCIN PRODUCTION. II.

X-RAY IRRADIATION ON THE PYRIDOMYCIN-PRODUCING STRAIN

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Pyridomycin is an antibiotic discovered by MAEDA, OKAMI, and UMEZAWA[1]. It inhibits the growth of *Mycobacteria* and Gram negative bacteria. As it was reported by MAEDA[2], pyridine nucleus is confirmed in this antibiotic. The pyridomycin-producing strain has been described by OKAMI as a new species, *Streptomyces albidofuscus*[3]. However, thereafter, the name of the species was changed to *Streptomyces pyridomyceticus* OKAMI *et* UMEZAWA.

The author studied, as reported in the previous paper[4], the rate of the lactose-utilizing mutants occuring after the ultraviolet irradiation. The mutation rate did not correspond with the killing rate. A mutant with the highest productivity of pyridomycin was obtained by the ultraviolet irradiation of the intensity with which the highest mutation rate was observed. Lactose-utilizing mutants, in general, exhibited higher productivity of the antibiotic than lactose-nonutilizing subcultures. A mutant No. 20-6-M 76 produced 420~528 mcg of pyridomycin in 1 ml of the shake-cultured broth. The amount of this production was about 10 times higher than that of the original culture.

As described in the previous paper, the mean resistance of spores to the ultraviolet ray was raised by the successive irradiation. It was considered that the change of the mutagenic agent would give a more successful result in getting a mutant with higher productivity of pyridomycin The mutant No. 20-6-M 76 was irradiated by X ray. The irradiation of the soft X ray was found to be weak in the killing effect and any strain having the higher productivity was not obtained. Irradiation of the hard X ray to the lactose-utilizing mutant induced a lactose-nonutilizing mutant. This lactose-nonutilizing mutant was similar to the original culture in the utilization of other carbohydrate and the productivity of pyridomycin. A strain, which was obtained from the spores of the strain No. 20-6-M 76 by X ray irradiation and utilized lactose, had higher productivity of pyridomycin than the parent culture. This strain, being different from the original culture, utilized mannose, mannitol, lactose, dulcitol, inositol, inulin, salicin, and tryptophane as the carbon source. These studies are presented in this paper.

Materials and Methods

The strains studied: The original culture producing pyridomycin was numbered as No. 451-A 8. After the first irradiation of ultraviolet ray to the original culture, the strain No. 20-6 with the higher productivity of pyridomycin was obtained. The ultraviolet irradiation to the strain No. 20-6 gave the strain No. 20-6-M 76. This strain was treated by X-ray and the strain No. M76-7X-119 producing from 700 to 800 mcg/ml of pyridomycin was obtained. The X ray irradiation to the strain No. M 76-7 X-119 gave the strain No. M76-7X-119-24, which utilized lactose and had the high productivity of pyridomycin, and the strain No. M76-7X-119-96 which did not utilize lactose and was low in the productivity of pyridomycin. These cultures were studied on their carbohydrate utilization and amino acids utilization as carbon sources.

The irradiation of X-ray: The spore suspension was placed on CZAPEK-DOX agar plates, being adjusted to about 100 spores per plate, and irradiated and the plate was incubated at 27°C. The colonies which grew on the plate were tested for the production of pyridomycin. The irradiation of soft X ray was made by Dr. KATŌ, Keio University. It was irradiated at the distance of 3 cm from the focus for 2, 5, 7, 10 or 20 minutes. The hard X ray (55 kV and 4 mA) was irradiated at Scientific Research Institute: 1800 r was irradiated per minute, and in total 30.000, 70,000 or 120,000 r was irradiated.

The examination of lactose and mannitol utilization of the irradiated spores: According to LEDERBERG and LEDERBERG[5], 4 days after the incubation of the irradiation plate, the replica plates were made on lactose (1%) CZAPEK-DOX agar and mannitol (1%) CZAPEK-DOX agar. The colonies on the replica plates were counted after the incubation at 27°C.

The examination of pyridomycin production: The cultures to be tested were shake-cultured in the following medium; glucose 2.5%, soybean meal 1.5%, NaCl 0.25%, KCl 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, K_2HPO_4 0.3%, $Na_2HPO_4 \cdot 12H_2O$ 0.3%, adjusted to pH 7.0. Twenty five ml of the medium was placed in a flask of 100 ml volume and shake-cultured at 27°C for 6 days. The concentration of pyridomycin in the broth filtrate was measured by the cup method with *Mycobacterium* 607. Pyridomycin in the mycelium

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mass was extracted into acetone, and, after evaporation of the solvent, it was dissolved in phosphate buffer at pH 6.0. The potency was determined by the cup method.

The examination of carbohydrate utilization: As the basal medium, CZAPEK-DOX salt agar was used, and the various carbohydrates shown in Table 2 was added at 2.0%. The cultures to be tested were streaked on the plate and incubated at 27°C for 7 days, and the growth was examined.

The examination of amino acids utilization as the carbon source: As the basal medium, CZAPEK-DOX salt agar was used. Various amino acids shown in Table 3 were added at 0.5%, and the growth of the cultures on the plates was examined after the incubation at 27°C for 7 days.

Results

The strain No. 20-6-M 76 was stable in the productivity of pyridomycin. In 6 experiments, the highest potency in the broth during shaking culture fluctuated between 406 mcg/ml and 482 mcg/ml. The irradiation of the soft X ray exhibited only very weak killing effect. The survival rates of spores after the irradiation for 2, 5, 7, 10, and 20 minutes were 83, 78, 72, 44, and 40% respectively. Fifty spores of each case were arbitrarily selected and tested for the production of pyridomycin. They were similar to the parent culture No. 20-6-M 76, and any strain with the higher productivity of pyridomycin was not obtained. The strain No. 20-6-M 76 was a lactose-utilizing mutant. The irradiated plates were tested by the replica method for the occurrence of lactose-nonutilizing mutant. None of the lactose-nonutilizing mutants was found after the irradiation of the soft X ray to the strain No. 20-6-M 76.

The results of the irradiation of hard X ray to the spores of the strain No. 20-6-M 76 is indicated in Table 1. Among 122 survived spores after the irradiation of 70,000 r, a mutant which did not utilize lactose and mannitol was found by the replica method. Two cultures among 41 spores survived after the irradiation of 120,000 r did not utilize lactose and mannitol. The production of pyridomycin of each culture obtained from the survived spores is indicated in Fig. 1. The strain No. M76-7X-119 obtained by the irradiation of 70,000 r to the strain No. 20-6-M76, produced 738 mcg/ml of pyridomycin. Three lactose-nonutilizing mutants produced 75~110 mcg/ml of pyridomycin, and this productivity was much lower than that of the mean productivity of the lactose-utilizing cultures shown in Table 1.

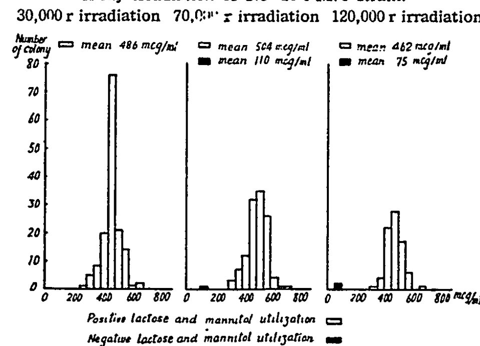
Table 1. Results of X Ray Irradiation to the Strain No. 20-6-M 76

X ray irradiated r	Number survived	Number irradiated	Killing rate %	No-lactose utilizing mutant	Mean of pyridomycin production of the survived strains*
0	1226	1226	100	0	453
30,000	148	1226	12.2	0	486
70,000	122	1226	10.0	1	504
120,000	41	1226	3.4	2	462

* The lactose-nonutilzuing mutants were removed from the result.

Fig. 1. Pyridomycin production of the strains obtained by X-ray irradiation of No 20-6-M76 strain.

30,000 r irradiation 70,000 r irradiation 120,000 r irradiation



The strain No. M76-7X-119 was irradiated by 70,000 r. Ten percent of spores irradiated survived and one among the survived did not utilize lactose and mannitol. The lactose-nonutilizing mutant No. M76-7X-119-96 produced 96 mcg/ml of pyridomycin. The mean of the production of pyridomycin by the lactose-utilizing culture obtained after the irradiation was 521 mcg/ml. The strain No M76-7X-119-24 which utilized lactose and mannitol and obtained by the irradiation of 70,000 r to the strain No. M76-7X-119 produced 786 mcg/ml of pyridomycin.

The carbohydrate utilizations of the strain No. 451A8 (the original culture), the strain No. 20-6 (derived from the strain No. 451A8 by ultraviolet irradiation), the strain No. 20-6-M76 (derived from the strain No. 20-6 by ultraviolet irradiation), the strain No. M76-7X-119 (derived fram No. 20-6-M76 by

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X ray treatment), the strain No. M76-7X-119-24 (derived from No. M76-7X-119 by X ray treatment) are shown in Table 2. Also the carbohydrate utilization of No. M76-7X-119-96, which was derived from No. M76-7X-119 by X ray treatment and which, being different from the parent strain, did not utilize lactose and mannitol, is also indicated in the same table. In Table 3 the utilization of amino acids of these strains are indicated.

Table 2. Utilization of Carbohydrates

Carbohydrates	Strains					
	No. 451A8	No. 20-6	No. 20-6-M76	No. M76-7X-119	No. M76-7X-119-24	No. M76-7X-119-96
Glycerol	⧻	⧻	⧻	⧻	⧻	⧻
L-Arabinose	+	⧻	⧻	⧻	⧻	⧺
D-Xylose	⧺	⧻	⧻	⧻	⧻	⧻
L-Rhamnose	−	±	+	+	⧺	±
Glucose	⧻	⧻	⧻	⧻	⧻	⧻
Fructose	⧺	⧻	⧻	⧻	⧻	⧺
Mannose	−	−	−	+	⧺	±
Galactose	⧺	⧻	⧻	⧻	⧻	⧻
Sorbitol	−	±	±	±	+	−
Mannitol	±	⧻	⧻	⧻	⧻	−
Dulcitol	−	±	±	+	⧺	−
Inositol	−	−	−	±	⧺	−
Inulin	−	−	−	+	⧺	−
Maltose	⧺	⧻	⧻	⧻	⧻	⧺
Sucrose	⧺	⧻	⧻	⧻	⧻	⧺
Lactose	−	⧺	⧻	⧻	⧻	−
Raffinose	−	±	+	+	+	−
Dextrin	⧻	⧻	⧻	⧻	⧻	⧻
Starch	⧻	⧻	⧻	⧻	⧻	⧻
Salicin	−	−	−	±	⧺	−
Aesculin	+	⧺	⧺	⧺	⧻	−
Na-acetate	−	−	−	−	±	±
Na-succinate	−	±	±	±	±	−
None	±	±	±	±	±	±

Table 3. Utilization of Amino Acids as the Carbon Source

Amino acids	Strains					
	No. 451A8	No. 20-6	No. 20-6-M76	No. M76-7X-119	No. M76-7X-119-24	No .M76-7X-119-96
Alanine	+	+	+	+	+	+
Leucine	±	+	+	+	⧺	+
Glutamic acid	+	+	+	+	+	+
Aspartic acid	+	+	+	⧺	⧻	+
Cystine	−	+	+	⧺	⧻	−
Cysteine	−	+	⧺	⧺	⧻	−
Lysine	±	⧺	⧻	⧻	⧻	+
Arginine	±	+	+	+	+	⧺
Histidine	−	+	+	+	⧺	−
Tryptophane	−	−	−	⧺	⧺	−
Proline	+	+	+	+	+	+
Asparagine	+	+	+	+	+	+
Hydroxyproline	−	+	⧺	⧺	⧻	±
None	±	±	±	±	±	±

No growth with glycine, α-aminobutylic acid, valine, nor-valine, serine, phenylalanine, tyrosine, methionine and ornithine.

The strains No. 20-6 and No. 20-6-M76 which had the higher productivities of pyridomycin than the strain No. 451A8 differed from the latter in the utilization of lactose and mannitol. The strains No. M76-7X-119 and No. M76-7X-119-24 which had higher productivities than the above strains differed from the strain No. 451A8 in the utilization of rhamnose, mannose, dulcitol, inulin and salicin. It is interesting that the strains which were obtained by the successive ultraviolet and X ray irradiations and exhibited

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the high productivity of pyridomycin utilized more widely the carbohydrates as the carbon source. The same was observed, as shown in Table 3, in the case of the utilization of amino acids as the carbon source. The strains No. M76-7X-119 and No. M76-7X-119-24 grew on CZAPEK-DOX salt agar with tryptophane. On the other hand, the lactose-nonutilizing strain No. M76-7X-119-96 which was derived from the strain No. M76-7X-119 was similar to the original culture in carbohydrate and amino acid utilization as the carbon source.

The production of pyridomycin of the original strain, the strain No. 20-6-M76, the strain No. M76-7X-119-24, and the strain No. M76-7X-119-96 were comparatively studied and the results are shown in Table 4.

Table 4. Production of Pyridomycin by Shaking Culture

Strains		2nd day		3rd day		4th day		5th day	
		pH	mcg/ml	pH	mcg/ml	pH	mcg/ml	pH	mcg/ml
No. 451A8	Filtrate	5.8	42	5.6	56	5.4	28	—	—
	Mycelium		21		20		18		
	Total		63		76		46		
No. 20-6-M76	Filtrate	5.6	300	5.5	428	5.4	380	—	—
	Mycelium		114		100		99		
	Total		414		528		479		
No. M76-7X-119-24	Filtrate	5.6	510	5.8	630	6.6	786	7.0	565
	Mycelium		127		110		109		96
	Total		637		740		895		661
No. M76-7X-119-96	Filtrate	5.4	52	6.0	81	6.6	90	7.0	60
	Mycelium		18		21		17		18
	Total		70		102		107		78

Summary

The strair No. 20-6-M76 which had been derived from the original strain by the successive ultraviolet irradiation was irradiated by X ray. The irradiation of 70,000 or 120,000 r gave a mutant whicb was similar to the original culture in the carbohydrate and amino acid utilization as the carbon source. The lactose-nonutilizing culture was inferior to the lactose-utilizing culture in the production of pyridomycin. The mutants which were obtained by X ray irradiation and had the high productivity of pyridomycin were different from the original culture in their utilization of rhamnose, mannose, dulcitol, inulin, salicin and tryptophane. They utilized more widely various carbohydrates and amino acids as the carbon source.

Here I should like to express my appreciation to Dr. H. UMEZAWA, the chief of our division, and Prof I. YAMASAKI of Kyushu University for their kind advices and directions.

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STUDIES ON GROWTH PROMOTION BY ANTIBIOTICS. I

EFFECTS OF CHLORTETRACYCLINE ON GROWTH

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MAPSON, BYERLY, *etc.* in 1932 discovered that, even when known nutriments and vitamins needed for animal growth have been given in full dosages, the growth is further promoted by an unidentified factor which is active in minute amounts. This factor they have named "animal protein factor" (A.P.F.). Further studies by BYERLY[1], PATTON[2], WRIGHT[3], RUBIN[4], ZUCKER[5], LANDINGHAM[6], and others found that A.P.F. is not contained in vegetable feeds, but in meat scraps, fish extract, liver extract, and feces of cattle and chicks. Active A.P.F. has also been detected by SHORB, STOKSTAD, RICKES STEPHENSON and others in the culture filtrate of microbes.

Because A.P.F. contains vitamin B_{12}, the promotion of animal growth by A.P.F. was initially ascribed to vitamin B_{12}. NEUMAN[7], ANDERSON[8], and others noted that swine reared on a diet supplemented with vitamin B_{12} in one month had a gain in weight 2 to 3 times of that of the controls kept without vitamin B_{12} supplementation of diet. BLACK[9] stated that growth promotion in white rats administered with vitamin B_{12} may partly be due to the stimulation of appetite by the vitamin. Administration of vitamin B_{12} to chicks right after hatching was found by OLEASE[10] to reduce their mortality rate. SAKAI has discovered vitamin B_{12} produced as an additional substance in the fermentation culture media of chlortetracycline, and streptomycin. A similar phenomenon has been described by CANNON. A conclusion was thereupon drawn by LOOSLI[11], HUNTER[12], REISTER[13], LILLIE[14], NICHOL[15], EMERSON[16], JOHNSON[17], NESHEIM[18], LEUCKE[19], and others that the effectiveness of A.P.F. is on the whole ascribable to vitamin B_{12}.

STOKSTAND[20][21], however, later found that chicks whose feeds were supplemented with chlortetracycline (Aureomycin) or *Streptomyces aureofaciens*, the source of chlortetracycline, had more promoted growth than could be attributed to vitamin B_{12} in the microorganism. COUCH[22], LILLIE[23], NICHOL[24], LUISE[25], COMB[26], and others have found that in A.P.F., besides vitamin B_{12}, there is an unidentified growth promoting factor with more powerful action of growth-stimulation than by vitamin B_{12} alone. Experiments by CUNHA[27] on swine and by MCGINNIS on turkeys noted little or no effect on growth by diets supplemented with vitamin B_{12} alone, but evident stimulation of growth by whole A.P.F. added to the diets. RICHARDSON[28], AKTINSON[29], SORB[30], HOFFMANN[31], MILLER[32], EDWARD[33], JUKES[34], LUECKE[35], and others have found that growth-promoting action is present in antibiotics themselves rather than in vitamin B_{12}. STOKSTAD[21] described that the growth-promoting effect of chlortetracycline has practically nothing to do with the addition of vitamin B_{12} and that rise in mortality rate coming from vitamin B_{12} deficiency can be prevented by adding chlortetracycline to the diet

We have studied how chlortetracycline promotes the growth of mice and causes changes in their body composition.

I. Effects of Chlortetracycline on Gain in Weight of Mice

Material and Method

(1) Experimental animals: Twenty mice each weighing about 9 g were used, dividing them into 4 groups of 5 animals each.

(2) Basal diet: A mixture of cereal powder 85% and fish meal 15%, pasted with water into dumplings, was used for basal diet.

(3) A.P.F: A.P.F. used in the experiment was Aurofac supplied by Lederle. It is in a powder form and each 1 g contains 26.8 mg of chlortetracycline and 4.2 mcg of vitamin B_{12}.

(4) Experimental procedure: Each of the 4 groups of mice was kept in a different cage. Group 1 as control was given only the basal diet; Group 2, the basal diet supplemented with 0.1% Aurofac; Group 3, the basal diet added with 0.3% Aurofac; and Group 4, the basal diet plus 0.8 mg/g chlortetracycline (Aureomycin). All the groups were kept for 6 weeks.

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Results

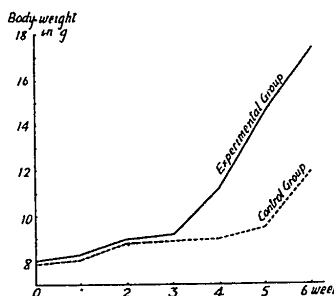
The body weight in Groups 1 to 4 at the beginning of the experiment was about equal, averaging 9.0, 9.4, 8.8 and 8.9 g, respectively.

Difference in body weight began gradually to appear with the progress of the experiment. The group later than the others to gain weight was Group 1, followed by Group 2. There was practically no difference between Groups 3 and 4 which registered the best growth. The difference in weight gain was the most marked during the 3rd to 4th weeks, the average weight for Groups 1 and 2 being 12.4 and 14.9 g, respectively, and for Groups 3 and 4 being 15.8 and 16.4 g, respectively. When later the mice had reached their maturity, no further increase in weight was to be seen, and Groups 1 and 2 retarded in growth gradually caught up with Groups 3 and 4, reducing the difference that had existed between Groups 1 and 2 and Groups 3 and 4.

Table 1. Body weight gain (mice)

Group	Feed	Before experiment	Average weight in g					
			1st week	2nd week	3rd week	4th week	5th week	6th week
I	Basal diet only	9.0	10.1	11.3	12.3	12.4	14.9	17.1
II	Supplemented with 0.1% Aurofac	9.4	11.4	12.4	14.2	14.9	16.1	16.3
III	Supplemented with 0.3% Aurofac	8.8	11.9	14.1	15.4	15.8	17.4	17.7
IV	Supplemented with 80 mcg/g chlortetracycline	8.9	11.8	14.3	16.2	16.4	17.2	17.8

Fig. 1. Weight curve of mice



II. Changes in Bone Weight

Material and Method

(1) Experimental animals: Fifteen mice each weighing about 8 g were used, dividing them into 2 groups.

(2) Basal diet: A mixture of wheat flour 50%, soy bean oil 10%, fish meal 15%, and whole milk powder 25%, was supplemented with multiple vitamin powder 0.5%, and pasted with water into dumplings, which were given as basal diet. Each gram of multiple vitamin powder contained vitamins in the following amounts: 1,250 units vitamin A, 0.5 mg vitamin B_1, 0.75 mg vitamin B_2, 18.75 mg vitamin C, 100 units vitamin D_2, 5.0 mg nicotinic acid amide, 0.25 mg calcium panthotenate, 0.05 mg vitamin B_6, and 0.25 mg folic acid.

(3) Experimental procedure: Seven control mice were given the basal diet only, and 8 experimental mice the basal diet supplemented with 0.2 mg/g of chortetracycline. Each group was kept in a separate cage for 20 days. All the mice were sacrificed after 20 days, by chloroformization. The viscera and skin were removed, and the muscles cling to the bones were also removed elaborately. Then, by sagittal incision along the meridiam line of the skull, the intracranial contents were removed. The skeleton was put into a 0.4% aqueous solution of caustic soda, and after heating for 20~30 minutes on a water bath at 40~50°C, it was washed well in funning water, the muscles still left on the skeleton was further removed in the water. This procedure was repeated until the skeleton was completely stripped. The water was then thrown away. A small amount of pure alcohol was added to the skeleton and caused to evaporate on a water bath. The skeleton was dried by heating for 1 hour at 90°C, and then kept in a drying apparatus until its constant weight was weighed.

Results

At the beginning of the experiment, there was practically no difference in average body weight between the control and experimental groups, which averaged 7.9 and 8.0 g, respectively. After 20 days, the former increased to 10 g, a gain of 2.1 g, and the latter to 12.6 g, a gain of 4.6 g. The bone weight showed practically no difference between the former group and the latter, averaging 0.30 g and 0.31 g, respectively. The body weight less the weight of the bone may be considered to represent the weight of soft tissues and water content, which may be regarded much larger in the experimental group than in the control (Table 2).

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Table 2. Body composition of mice (Part 1)

Feed	Number of mice	Weight before experiment	Weight 20 days later	Bone weight	Soft tissues and water content
Basal diet only	7	7.9 g	10.0 g	0.30 g	9.70 g
Supplemented with 0.8 mg/g chlortetracycline	8	8.0	12.6	0.31	12.29

III. Quantitative Analysis for Water Content and Fat

Material and Method

(1) Experimental animals: Ten mice weighing 9∼10 g were used.

(2) Basal diet: Same as that used in the preceding experiment.

(3) Experimental procedure: Five mice as controls were fed on the basal diet only; 5 were given the basal diet plus 0.2 mg/g of chlortetracycline. Each group was kept in a separate cage, for 15 days. After the 15 days, feces right after excretion were made into a paste with a amall amount of distilled water, and their pH was measured with Toyo Roshi Company's test paper for the concentration of hydrogen ions. After a 5-hour fasting, they were sacrificed by choloroformization, and weighed. The viscera were removed, the other parts of the body minced, and all those pooled and put into a grinding dish. With a small addition of pure alcohol, the grinding dish was placed on a water bath for evaporation. Adding alcohol again into the contents of the grinding dish before drying, they were broken up as fine as possible by manipulating with a glass rod. After evaporation, they were dried for 24 hours at 60°C, weighed, and put to quantitative analysis for fat by the KUMAGAWA-SUDO method.

Results

The average body weight which before the experiment was 9.6 g for the control group and 9.7 g for the experimental group, was after 15 days found increased to 11.5 g. and 12.5 g, respectively, the latter group showing thus a higher increase than the former. The pH value of the feces was 6.2 for both groups, with no difference noted. Approximately the same between the two groups was also the water content of the body. Significant difference, on the contrary, was observed in the amount of neutral fat, which was 4.2% in the controls the 5.1% in the experimental groups (Table 3).

Table 3. Body composition of mice (Part 2)

Feed	Number of mice	Weight before experiment	Weight 15 days later	Water content	Neutral fat	pH of feces
Basal diet only	5	9.6 g	11.5 g	68.0%	4.2%	6.2
Supplemented with 0.2 mg/g chlortetracycline	5	9.7	12.5	67.9	5.1	6.2

IV. Quantitation of Protein

Material and Method

(1) experimental animals: Thirteen mice weighing about 8 g were used.

(2) Basal diet: Same as in the preceding experiment.

(3) Experimental procedure: A control group of 6 mice was maintained on the basal diet only, and an experimental group of 7 mice was fed on the basal diet plus 0.2 mg/g of chlortetracycline keeping each group in an individual cage, for 20 days. After a 5-hour fast, they were sacrificed by the administration of chloroform. After mincing, drying, and weighing as was done in the preceding experiment, the material was transferred into a separating funnel. Adding ether therein, fat was extracted as much as possible. The material together with about five times as much of concentrated sulfuric acid then was heated and dissolved. A part of the solution was put to the micro-KJELDAHL determination (PARNAS' modified method) for nitrogen, to work out the body protein level.

Results

Both groups before the experiment were of equal body weight, 8.0 g. Twenty days later, the experimental group showed a higher gain in weight than the control. As in the preceding experiment, there was no difference in water content between the experimental and control groups. Protein was

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19.7% in the control groups and 20.6% in the experimental group; the latter was thus slightly richer in protein than the former. The difference, however, could not be taken to be statistically significant (Table 4).

Table 4. Body composition of mice (Part 3)

Feed	Number of mice	Weight before experiment	Weight 20 days later	Protein	Water content
Basal diet only	6	8.0 g	10.3 g	19.7%	69.5%
Supplemented with 0.2 mg/g chlortetracycline	7	8.0	12.2	20.6	68.5

V. Changes in Neutral Fat and Phosphorus-containing Lipoid of the Liver

Material and Method

(1) Experimental animals: Sixteen mice weighing about 8 g were used, divided into 2 groups.

(2) Basal diet: A high-fat diet of the following composition was given: Wheat flour 60%, soy bean oil 30%, and fish meal 10%. The neutral fat content of this diet was analytically determined as 35.5%. Multiple vitamin powder was added 0.5% as in the preceding experiment.

(3) Experimental procedure: Eight mice, as controls, were fed only on the basal diet; another 8 were maintained on the basal diet supplemented with 0.3 mg/g of chlortetracycline. The two groups were each kept in a separate cage, for 20 days. After a period of 20 days and 5-hour fasting, the mice were sacrificed by administration of chloroform. Promptly thereafter, the liver was removed and weighed. A part of the livers of 5 mice from each group was fixed with formalin, embedded in carbowax, and put to fat-staining with Sudan III. The rest of the livers was analysed for fat. The livers of the remaining mice (2 and 3 of the control and experimental groups, respectively) were emulsified with 9 times as much water in a homogenizer. The emulsion was put to photoelectric colorimetry for phosphorus-containing lipoid by the aminonaphtolsulfonic acid method.

Results

For about one week after beginning the experiment, no particular difference in the general condition of the mice was noted between the two groups. From about the 10th day onward, the mice of the control group had dirty, bristled hair, and apparently lost strength and appetite. On the 15th day, one of them died. The experimental group, on the contrary, had normal growth, with no mortality during the entire period of experiment. While the weight of liver was not particularly different between the two groups, fat weighed more in the control group, especially indicating 6.2% of neutral fat, the chortetracycline group showed neutral fat to be only 4.7%. There was scarcely any difference between the two groups as regards the amount of phosphorus-containing lipoid (Table 5)

Table 5. Mice liver analysis

Feed	Number of mice	Liver weight 20 days later	Total fat	Neutral fat	Phospholipid mg/g
Basal diet only	7	0.65 g	7.9%	6.6%	27.7
Supplemented with 0.3 mg/g chlortetracycline	8	0.68	6.2	4.7	27.4

Microscopic tissue specimens corroborated with the results of analysis. Four of the 5 control mice exhibited fat deposition in the vicinity of GLISSON's capsule at the periphery of the lobules; only 1 control presented no deposition of fat. Whereas, in the chlortetracycline group, 2 of 5 mice showed fat deposition of a very slight degree. In both groups, the fat deposition was noted only at the periphery of the lobules and not at their central parts. The deposited fat was present in the form of fat globules within the hepatic cells. None of the mice showed degeneration of liver cells, atrophy, cellular infiltration, proliferation of the interstitial connective tissue, hemorrhagic changes, or other abnormal picture.

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VI. Effect of Chlortetracycline in Malnutrition

Method and Material

(1) Experimental animals: Ten mice weighing about 8 g and poor in weight gain, were used, dividing them into 2 groups of 5 mice each.

(2) Basal diet: Same as that in Chapter IV.

(3) Experimental process: Each group was kept in a separate cage for 2 weeks, on the basal diet only. During the 2 weeks, both groups registered a low rate of weight gain, with no difference between the min gained weight. One of the 2 groups was then given the basal diet supplemented with 0.5 mg/g of chlortetracycline and the other group the basal diet only, both for 4 weeks while under observation.

Results

The average body weight before the beginning of experiment was 7.9 g for the control group, and 8.0 g for the experimental group. During the period of 2 weeks of the basal diet only, the average weight for the control group and the experimental group was 8.8 and 9.0 g, respectively. The difference between the two groups was thus negligible, both being poor in the rate of weight gain. From about the 3rd week after beginning of experiment (or the 1st week after the institution of chlortetracycline administration), weight gain in the experimental group became a little better. In the 6th week after the experiment began (or in the 4th week after chlortetracycline supplementation of the diet was started), the average weight of the experimental group was 17.4 g or nearly normal. Whereas, the control group was still in poor growth, the weight averaging 12.0 g (Table 6, Fig. 2).

Fig. 2. Weight curve of mice in malnutrition

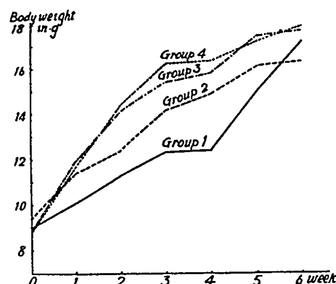

Table 6. Weight gain of mice in malnutrition

Feed	Before experiment	Week of experiment 1st	2nd	3rd	4th	5th	6th
Basal diet only	7.9	8.1	8.8	8.9	10.0	10.5	12.0
Supplemented with 0.5 mg/g chlortetracycline	8.0	8.3	9.0	9.2	11.2	14.6	17.4

Summary and Comment

Chlortetracycline added to the diet of very young mice did promote their growth more than the growth in control which coincided with what has been reported already by many workers. The reason for the less difference in weight gain of that of the controls from about the 5th week after the beginning of experiment, is because the mice has reached their maturity about that time and no further increase in body weight was naturally to be seen. This is considered to mean, as stated by COHEN[38], that the growth-promoting effect of the antibiotic can be observed only during the time the animals are very young.

Little or no difference between the chlortetracycline group and the chlortetracycline plus vitamin B_{12} group, is interpreted to the fact that what promotes growth is chlortetracycline, the effect of vitamin B_{12} on growth being little or nothing.

In order to know what component of the body was the most influenced during the marked gain in weight caused by chlortetracycline, analytical examination was made, distinguishing bone tissues from the other tissues of the body (soft tissues and water content.) Weight gain was thereby found higher in the latter. The latter's gain in weight was considered accountable for the gain in bodyweight. There was no appreciable difference in the water content between both groups; the water content was proportionate to the body weight. This finding is contrary to a statement by KNOEBEL[37] that the water content of the body is lowered by chlortetracycline and streptomycin. The neutral fat content in soft tissues was evidently higher in the experimental group than in the control group, which corroborates with the

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reports of KNOEBEL[37], LOTTE[38], WAINFAN[39], SINGER[40], and others. LOTTE[38] reported that the specific gravity went lower as the fat increased. As for the nitrogen metabolism, BROWN[41], BRANDE[42], BLACK[9], LOTTE[38], KNOEBEL[37], and others stated that it did not change following the administration of chlortetracycline. In Japan, on the contrary, TAKAI[43] and OHTA[44] have described rise in nitrogen accumulation following chlortetracycline administration. In such a case as this, rise in body protein level must be conceivable. But in our present studies, while the absolute amount of protein did increase, its ratio on body weight showed no difference in either group.

ICHIDA[45] and many other workers have described that fatty liver develops in animals kept on a high-fat diet. BEESTON[46] states that in such a cass as that, the liver is often found in hypertrophy. In our present studies, the weight of the liver did not register any particular change following the administration of chlortetracycline. In a group maintained on a high-fat diet only, the liver fat level was found higher than normal, and fat deposition was detected by histological procedures, also. In the chlortetracycline group, on the contrary, the liver fat level was within the normal range.

BAXTER[47] reported that chlortetracycline is effective in the prophylaxis of fatty liver due to choline-deffecient diets. GYOERGY[48] likewise described that chlortetracycline administration prevented the necrosis and cirrhosis of the liver. These statements appear to coincide with the findings obtained in our present studies. The question not yet solved is whether the prevention of fat deposition in the liver by chlortetracycline indicates that chlortetracycline itself has lipotropic activity or that chlortetracycline makes choline more active or that chlortetracycline reduces the need for vitamin B_{12}. While BAXTER[47] has stated that the administration of chlortetracycline increases the level of choline in the feces, there was no difference between the experimental and control groups under the present studies as regards the level of phosphorus-containing lipoid in the liver. This suggests that the process of phosphate formation in the liver is not particularly influenced by chlortetracycline administration. The better growth in the chlortetracycline group than in the control may suggest, as stated by SLINGER[40], that fat tolerance is higher in the former group.

The above findings came from the results of experiments with normal mice. Administration of chlortetracycline to mice of lower weight gain and considered to be of malnutrition began likewise to show a marked improvement in body weight in or about the 2nd week after the institution of chlortetracycline administration. As their food intake prior to the chlortetracycline administration was sufficient, the marked improvement in their weight after chlortetracycline supplementation of diets could be attributed to chlortetracycline.

Conclusion

Observation was made how chlortetracycline added into mice's diet influenced their growth. Changes in their body components were also studied.

1. Supplementation of the diet with chlortetracycline brought about a higher rate of bodyweight gain than in the controls.
2. No difference in average bone weight was observed between the experimental group and the control group.
3. Chlortetracycline caused rise in the absolute amount of water content and protein in the experimental group, but no change in their ratio on body weight. Body-fat level was increased.
4. The chlortetracycline group, even on a high-fat diet, had a lower liver fat level than that in the control group.
5. Even in the case of malnutrition with very low rate of weight gain, chlortetracycline administration caused a marked increase in body weight.

The outlines of this report was read at the 1st general meeting of the Kinki chapter of the Japan Society of Chemotherapy in November, 1953.

The author wish to express his appreciation for the help and advice received from Prof. NAGAI.

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STUDIES ON GROWTH PROMOTION BY ANTIBIOTICS. II

RESULTS OF AUROFAC ADMINISTRATION TO INFANTS

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Since antibiotics were found to promote the growth of young animals, with no side reactions even when given over a prolonged period of time, antibiotics have come to be utilized for promoting growth in man, too.

ROBINSON[1] reported that twins and triplets given 50 mg/kg daily of chlortetracycline had better growth than the controls. IWAKAWA[2] gave premature infants 10 mg of oxytetracycline daily for 5 months from the 5th month of life, and observed better growth, without appreciable side effects, except that the stools became a little softer. TAKAI[3] and OHTA[4] described growth promotion in sucklings given Aurofac containing chlortetracycline.

We have conducted a series of experiment to observe how Aurofac administered to infants ranging in age from 20 days to 1 year and 5 months increase their weight. The results of the experiment are presented in this paper.

EXPERIMENTAL PROCEDURE

Experiment was conducted on 9 infants, 5 boys and 4 girls, from 20 days to 1 year and 5 months after birth. Most of the infants weighed less than the standard when the experiment was begun: their daily average gain in weight was also less than the standard.

Six of the infants in the 1st experimental group were observed for 10 to 30 days before the beginning of the experiment, they were given Aurofac (each gram of which contains 26.8 mg chlortetracycline and 4.2 vitamin B_{12}) 0.5~3.0 g daily, divided into 3 doses.

Breast-fed infants received a small amount of aqueous solution of each dose of Aurofac powder before breast-feeding; bottle-fed infants and mixed fed infants received a milk mixture with a dose of Aurofac powder before bottle-feeding. The administration of Aurofac continued for 15 to 65 days, keeping observation on the weight gain and general condition of the infants.

Three cases of the 2nd experimental group were given 1 g Aurofac daily for about 70 days. One infant equal in age, sex, feeding method and of as closely resembling environmental conditions as possible to each of the three cases, was used as controls.

One infant each of bottle-feeding, mixed feeding, and weaning stage, was observed for fecal pH and alterations in *E. coli* before the experiment and at 1 week after the institution of Aurofac administration. In examining the stools for *E. coli*, one loopful was obtained aseptically from three parts of the feces, and mixed with 100 cc of sterilized physiological saline solution. Of the above fluid, one loopful was taken and smeared on DRIGALSKI-CONRADI culture medium. Forty-eight hours later, the average number of *E. coli* colonies was determined.

RESULTS

Four of the 6 infants in the 1st experimental group had had lower initial weight and less weight gain than the standard. Another infant (H.N.) had weighed nearly the same as the standard, but had less gain in weight. Still another infant (Y K.) showed quite normal growth. For about 2 weeks after institution of Aurofac administration, there was no appreciable change among the infants. Five of the 6 infants subsequently began to show a gradual rise in weight, and approached the standard weight on completion of the experiment. The one infant whose growth was normal from the beginning showed no effect by Aurofac.

All the infants of the 2nd experimental group had been of lower weight than the standard. Two of the 3 infants given Aurofoc began to show a gradually marked gain in weight from about the 2nd week after its institution; and when the experiment ended, these 2 infants had nearly normal weights. The controls, on the contrary, kept low in weight gain, and their general condition appeared inferior to that of the infants of the Aurofac group (Table 2).

In the experimental groups 1 and 2, the infants given Aurofac not only exhibited significant rise in body weight but also seemed to have increased subcutaneous fat deposition. On the whole, fatness rather than growth promotion was more impressive in these infants.

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Table 1. Results of Aurofac administration to breast-fed infants. I.

Name	Age	Sex	Diet	Daily Aurofac dosage g	Number of days of observation	Weight before experiment kg	Weight on completion of experiment kg	Daily average gain in weight g
M. Y.	2 m.	♀	cow's milk	0.5 1.0	17 25 33	4.03 4.30 5.04	4.30 5.04 6.10	15.9 29.6 13.1
Y. K.	3 m.	♀	powdered milk	0.5 1.0	16 30 35	5.60 5.90 6.50	5.90 6.50 7.20	18.7 20.0 20.0
F. N.	1y. 5 m.	♂	mixed feeding	1.5 3.0	30 14 19	8.51 8.75 8.91	8.75 8.91 9.45	8.0 11.4 28.4
S. O.	25 d.	♂	mother's milk	1.0 1.0	20 34 24	2.65 2.95 3.50	2.95 3.50 4.25	15.0 16.2 30.0
H. N.	7 m.	♂	powdered milk	3.0 3.0	28 14 14	7.25 7.35 7.45	7.35 7.45 7.75	3.6 7.2 21.4
E. O.	20 d.	♀	mother's milk	1.0	10 15	2.53 2.60	2.60 3.09	7.0 32.7

Table 2. Results of Aurofac administration to breast-fed infants. II

Name	Age	Sex	Diet	Daily Aurofac dosage g	Number of days of observation	Weight before experiment kg	Weight on completion of experiment kg	Daily average gain in weight g
T. T.	3 m.	♂	powdered milk	1.0	70	5.15	6.25	16.0
S. N.					70	5.15	7.20	29.3
M. S.	3 m.	♀	powdered milk	1.0	68	4.38	5.55	17.2
K. T.					68	4.20	5.30	16.2
M. H.	40 d. (Twins)	♂	powdered milk	1.0	70	4.60	6.00	20.0
A. H.					70	4.40	6.30	27.1

Table 3. Changes in feces following Aurofac administration

Age	Diet	pH before administration of Aurofac	pH after Aurofac administration started	*E. coli* before administration of Aurofac	*E. coli* after Aurofac administration started
1 m.	Bottle feeding	6.8	6.6	30	7
2 m.	Mixed feeding	6.4	6.2	20	2
10 m.	Weaning stage	7.8	7.2	∞	35

Many of the bottle-fed and mixed fed infants began to approach the breast-fed infants in the character of feces 2 or 3 days after institution of Aurofac administration: the feces became softer and more yellowish. Feces of one infant each of bottle-feeding, mixed feeding and weaning stage were examined, the result of the findings were: Before Aurofac administration, fecal pH was the highest in the weaning infant, next in the bottle-fed infant, and the lowest in the mixed fed one. The number of *E. coli* was the highest in the weaning infant, and less in the other infants in the same order as above. One week after Aurofac administration was begun, the pH began to drop and *E. coli* began to decrease in all the infants (Table 3).

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DISCUSSION

Seven out of 9 infants administered with Aurofac exhibited increase in daily gain in weight. No effect of Aurofac administration was observed in one of the remained 2 infants, this was interpreted due to the fact that the infant had had normal weight and growth at the beginning of the experiment.

The gain in weight became remarkable from about the 2nd week after the institution of Aurofac administration. Similar experience as this has been described by OHTA[4] and TAKAI[3]. ITOGA[5] has, as a result of the LONG's test, noted a high incidence of adrenocortical dysfunction among infants of poor gain in weight. He has then reported on a close relationship between adrenocortical dysfunction and malnutrition. Such infants, on receiving Aurofac, would stimulate their adrenocortical function and would become nearer to normal, resulting in improved nutritional condition and marked gain in weight, as stated in Part 4.

In breast-fed infants, the intestinal contents tend to acidity, because their intestinal flora are mostly *Lactobacillus*, a small amount being *E. coli*. In bottle-fed infants, whose ratio of *Lactobacillus* to *E. coli* is just the reverse to the above, the intestinal contents tend towards alkalinity, as is generally known. BROWN[6] and GERSTLAY[7] hold the difference in intestinal flora to be one of the factors for difference in growth between the breast-fed and bottle-fed infants. EDWARD[8] has reported that oral administration of *Lactobacillus* to the bottle-fed infant causes the infant's growth to be similar to that of thebreast-fed one.

Various workers have reported that antibiotics decrease the number of *E. coli* in the intestinal tract and acidify the intestinal contents. That Aurofac tends towards the same result as that is known from the results of our present experiment. Improved nutritional status in bottle-fed and mixed fed infants might therefore be partly due to changes in intestinal flora and in intestinal pH. However, what may be conceived as the principal factors for their improved nutritional condition are, as shown by the results of the experiment described in Part 4, enlivened anterior lobe of the hypophysis, better functioning of the adrenal cortex, the consequent rise in synthesis of glycogen and rise in absorption and increased body store of fat.

CONCLUSION

Seven out of 9 infants were given 0.5 to 3.0 g of Aurofac daily for a period of about 2 months, and a marked rise in body weight was observed. The weight began to increase in about 2 weeks after institution of Aurofac.

Three of the infants were examined for intestinal *E. coli* and fecal pH. The Aurofac administration was thereby found to decrease *E. coli* and to shift the feces to acidity. This, however, is merely one of the factors for gain in weight. The results of experiment described in our last report indicate the weight gain to be ascribable to enlivened anterior lobe of the hypophysis and better functioning of the adrenal cortex, and the consequent rise in glycogen formation and fat absorption.

The outline of the present report was read at the occasion of the 6th meeting of the Clinical Committee of the Japan Antibiotics Research Association, on November, 1952.

Heartly appreciations are expressed for Professor NAGAI's kind directions and review of the draft-report.

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Trichomycin "Sanyo"

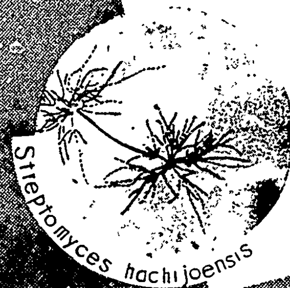
Indication:

Trichomonas vaginalis vaginitis
Moniliasis of vaginal

Supplied

Trichomycin Vaginal Tablets containing 50,000 units of trichomycin each.

Trichomycin Penicillin Vaginal Tablets containing 50,000 units of trichomycin and 50,000 units of crystalline penicillin G each.



Penicillin "Sanyo"

Crystalline Penicillin G
and
Various Penicillin
Preparations

Manufactured by
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Sole Agent
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PHARMACEUTICAL CO., LTD.
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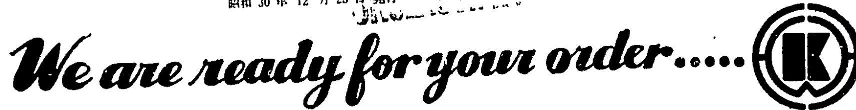

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PROGRESS OF ANTIBIOTICS IN JAPAN

by
Yukimasa Yagisawa,* Secretary General of
Japan Antibiotics Research Association, Tokyo

The research, production and application of antibiotics in Japan made a striking development during past 12 years.

The antibiotic research was first initiated in February 1944, when Buenos Aires correspondence reported the successful penicillin treatment of British Prime Minister Winston Churchil. The Penicillin Committee was then established under the auspice of Army Medical College, Tokyo No information was available at that time, and facilities for study were very dificient but the ardent efforts of the Penicillin Committee led to the success in producing the first home-made penicillin in the fall of the same year These preparations were used in the treatment of those wounded in the war or burned by air raid just before the end of war, though less in quantity and low in purity. The Penicillin Committee completely stopped its activity by the end of the war.

After the war, as a few companies started to manufacture penicillin and American penicillin was introduced through the occupation personals and black marchants, clinical investigation was proceeded. However, in April 1946, penicillin production was prohibited by the order of occupation forces. This event was a great blow to the manufacturers who initiated the production with much difficulty. and again the investigation was stopped. It was cleared afterwards that the occupation forces intended to raise the quality of Japanese penicillin with lower potency than that of America, and to produce enough penicillin for the treatment of infectious diseases including venereal For this object, a systematic investigation begun again under the auspice of Welfare Ministry, Japanese Government, in August 1946.

Penicillin Research Association—Japan Antibiotics Research Association since 1948 — was then established to promote the all round investigation on production and clinical application of antibiotics and to give the technical guidance to the manufacturing factories. Research Committees were set up assembling the scientists in bacteriology, agricultural chemistry, organic chemistry, pharmacology and clinical medicine who had concerned with Penicillin Committee, and newly including experts on physiology, veterinary medicine and chemical engineering. The research was supported by the grants from the Educational Ministry, and later by Welfare Ministry and Agricultural Ministry, which markedly accelerated the research progress of antibiotics. Excellent results were obtained first in the production and clinical application of penicillin, followed by those of streptomycin, and now the research is continuing with the aim at new antibiotics and animal feed.

Viewing the antibiotic studies in the world, early penicillin research was carried out in England and later important one almost in the United States. In Japan, the work was started 3 years later, and was holding 3 years' backwardness to the United States. Recently difference between the two countries has become less, and the research, especially on new antibiotics, is more advanced in Japan than in other countries.

Research on Penicillin Production

The investigation of penicillin production was begun with isolation of penicillin producing molds and their improvements. Penicillin Committee tested the strains obtained from all over the country, and selected p. 176 and p. 233 strains. These were used for the commercial production until the summer of 1947 with a yield as much as about 50 units (0 003mg) per ml of culture medium At this time, penicillin was produced by so-called surface culture method Culture liquid was transferred into glass bottle, in which a penicillin-producing mold

* visited Rangoon in Jan., 1955

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was inoculated and kept at 25°C. The surface culture method had disadvantages that a tremendous large number of bottles was required, and that if even a single bottle was contaminated in gathering culture medium, the produced penicillin was destroyed Thus in order to manufacture penicillin on commercial scale, a replacement with tank fermentation or submerged fermetation method was necessary Unfortunately, Japanese researchers failed to isolate the mold strain suitable for the submerged production

The strain for the submerged culture was first introduced by Dr J W Foster who was in charge of penicillin consultant in the General Headquarters for 4 months from November 1946 to March 1947 This was Q 176 strain which had been used in the United States for the submerged production. It was continuously improved by single spore isolation and induced mutation by X-ray, ultraviolet ray and mutagenic chemicals such as nitrogen-mustard, and now yeilds over 4,000 units (2 4mg) per ml of culture liquid Comparing the potency with that of Oxford researchers, more than 1,000 times' penicillin is being produced from the same amount of culture liquid

In viewing the research on penicillin producing strains, it is noteworthy that Dr K Arima, Assistant Professor in the Department of Agricultural Chemistry, Tokyo University, developed a new pigmentless saltant of Q-176 strain The original strain of Q-176 produced yellow pigment in culture medium, which caused difficulty of purification of penicillin This pigmentless saltant was adequate to overcome this difficulty and to increase the production

The culture media were constantly improved throughout surface and submerged fermentation for obtaining the medium with high yield and ease in purification At the beginning, material employed were by-products from agricultural and marine products because of food shortage

Fermentaion method was investigated mainly on submerged production. The first fermenter was designed by Dr Oyama and Dr Uchiyama of Tokyo Institute of Technology based on the blue print which Dr Foster brought in Japan Later it was improved and many commercial fermenters as large as 60-ton capacity were built based on experimental fermentation data The design of the first fermenter was so circumspective that it is still used in the experimental fermetation of new antibiotics.

The submerged production was started in spring 1946. But in order to maintain the output, the surface culture was continued The submerged culture is more suitable for large scale production than bottle culture But at the beginning, the yield was as low as 50 200 units per ml of culture liquid and sometimes no penicillin was produced from contaminations. Nowadays, the yield as well as fermentation technique increased to 4,000 units per ml. or more as the result of improvement of culture medium

During the period of Penicillin Committee, penicillin was recovered from culture liquid by activated carbon absorption and ether extraction Later, ether was substituted for acetone Carbon absorption method had various disadvantages: the procedure was so intermittent that unsuitable for large-scale production, the yield varied according to the quality of carbon, the solvent with low boiling point had a danger to cause a fire The solvent extraction method which came to be used widely thereafter had no such disadvantages, but must be proceeded rapidly at a low temperature to prevent destruction of penicillin The reason is that in transferring penicillin from culture liquid to solvent (butyl acetate), penicillin is easy to be destroyed in acidifying culture liquid The ejector devised by Dr Oyama solved this problem The combination of ejector with high-speed centrifuger made easy the treatment of large-amount culture medium, and, along with basic studies on solvent extraction, led to the rapid elevation of purification yield.

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The last step of penicillin purification is the process for obtaining penicillin crystalls by drying concentrated penicillin solution. The yield was as little as 20% in the early days. For drying penicillin, so-called freeze-drying method was used Freeze-drying equipment was accomplished by Dr. Nishina of Scientific Institute, a world-famous atomist, and drying conditions were studied by Dr Umezawa of National Institute of Health. Through their efforts the loss of penicillin by drying became almost none

The progress of penicillin-producing method remarkably favoured commerical production. Though the manufacturing facilities are not so enlarged, the output amounted as much as 46 million units in 1946, 1,380 million units in 1947, 29,000 million units in 1948, and 180,000 million units in 1949. And now about 200,000 million units are being produced monthly On the other hand, the production cost was rapidly reduced; it was 3930 Yen per 10,000 units in 1946 (not less than 60,000 Yen when corrected by price index) and now not more than 40 Yen

Progress in Penicillin Preparations

In 1946, penicillin was supplied as an amorphous powder. The expiration date was as short as 1 month even in refrigerator In order to increase the stability of penicillin, it was necessary to minimize the moisture content With the progress of above-mentioned freeze-drying method, the moisture content reduced rapidly According to the national assay, in spring 1947, moisture content of not more than 2 5% was scarce, and average potency was 200 units per mg. In the autumn 1948, all the products were of not more than 2 5% moisture, and penicillin and the purity was markedly improved; crystalline penicillin became available Crystalline is stable for 6 days at 100°C and for 4 years if kept at room temperature.

New kinds of penicillin preparation have being divised. In autumn 1948, penicillin in oil and wax which suffices for once-a-day injection was developed, followed by procaine penicillin for aqueous injection and penicillin tablet in autumn of the same year, etc. These preparations were first devised in the United States, but the clinical experiments of the sample as well as the study for maintaining the stability were carried out in Japan. Pyrimidine penicillin was a new penicillin preparation developed in Japan

Investigation on Penicillin Therapy

The clinical results of penicillin treatment in the United States were re-examined in Japan as t[illegible] [illegible]re introduced. However, most efforts in early days were exerted to obtain the greatest effects with the smallest quantity and to improve the quality of preparations. The most important subject in penicillin treatment is the in-vivo concentration of penicillin, namely, absorption, distribution and excretion. It was estimated by the superposing method devised by Dr. Torii of Physical Therapy Department of Tokyo University. This method is succesfully applied to determine the concentration of other antibiotics in body fluids.

Clinical investigation of penicillin was performed by cooperation of clinicians of various fields and its results were published in medical journals without delay In 1949, "Standard of Penicillin Therapy" was published by Japanese Medical Association and facilitated the wide-spread use of penicillin. As the results, the death rate of pneumonia dropped from the second rank in 1947 to below the sixth in 1948 and thereafter Concurrently the cure rate of other penicillin indications remarkably increased and duration of treatment was shortened. The effect of penicillin for the prevention of complications or for prophylaxis of infections was proved to be remarkable Occurrence of post-pnemonia pyothrax or puerperal fever became almost none.

Progress in Streptomycin Investigation

In spring 1946, Dr Umezawa isolated a streptomycin-producing strain, and in summer 1947, research on the production was initiated In

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spring 1948, wide clinical experiments of tuberculosis were carried out with imported streptomycin. The commercial production of streptomycin was started in summer 1949 Utilizing the experiences in penicillin production, research was proceeded on the strains, fermentation, extraction and processing and resulted in a rapid advance in the yield and quality of streptomycin Streptomycin is now produced 2.5 tons a month.

The important projects encountered during the research on streptomycin production were the countermeasure for actinophage which destroyed the mycelium during cultivation, the extraction by ion exchange resin absorption, and the reduction method in dihydrostreptomycin production. These were solved through the extensive investigations of Dr Aiso, Putrefaction Institute, Dr S.Umezawa, Keio University, and Dr. Sumiki, Tokyo University

A success in the tuberculosis therapy with streptomycin is evident from the fact that the death rate of tuberculosis fell down from the first rank to the fifth during past 4 years, and the death of young men which was at the top in the past became almost none The research for obtaining highest cure rate with minimum sidereaction is still continuing. The most advanced method of streptomycin treatment is the combination of streptomycin injection twice a week and oral administration of PAS and or INAH every day This schedule is far superior to single daily injection of streptomycin in the respect of preventing the development of resistant tubercle bacilli and minimizing side reaction to nervous system.

Streptomycin administration for other than tuberculosis was investigated under cooperation of physicians of various departments. The results are adopted in "Guide to Antibiotic Therapy" published by Japan Medical Association in January 1953

Research for New Antibiotics

The start to search for new antibiotic substances in Japan was far later than in the United States because all the researchers had engaged in the investigation of penicillin and streptomycin However, many new antibiotic substances were developed recently among which the followings are commercially produced and sold trichomycin by Dr Hosoya, Institute for Infectious Diseases, leucomycin by Dr Hata, Kitasato Institute, gramicidin J by Dr Otani, Osaka City University, aureothricin and sarkomycin by Dr Umezawa, National Institute of Health.

Research on new antibiotics was started with the aim for obtaining substances effective against penicillin and streptomycin-unsusceptible pathogens such as rickettsia, virus, protozoa and fungus. In the United States, chloramphenicol was discovered in 1947, chlortetracycline in 1948, and oxytetracycline in 1950 These antibiotics are effective against rickettsial diseases such as typhus or tsutsugamushi-disease, and in infections caused by large viruses such as trachoma and atypical pneumonia Clinical experiments were carried out on these antibiotics using imported preparations, and the results are adopted in "Guide to Antibiotic Therapy" After the development of these broad spectrum antibiotics, successful results are obtained in the treatment of typhus, typhoid fever, dysentery or trachoma. Tsutsugmushi disease with high death-rate became more mild than common cold.

Since Dr Umezawa discovered streptothricin B, the same substance as neomycin described by Dr Waksman thereafter, in 1947, Japan retained its backwardness in the research especially on new antibiotics. As soon as erythromycin and carbomycin were discovered in the United States, leucomycin was reported in Japan which provided the same activity and similar chemical structure as such antibiotics. In the research of antifungal and antiprotozoic substances, trichomycin was reported from Japan

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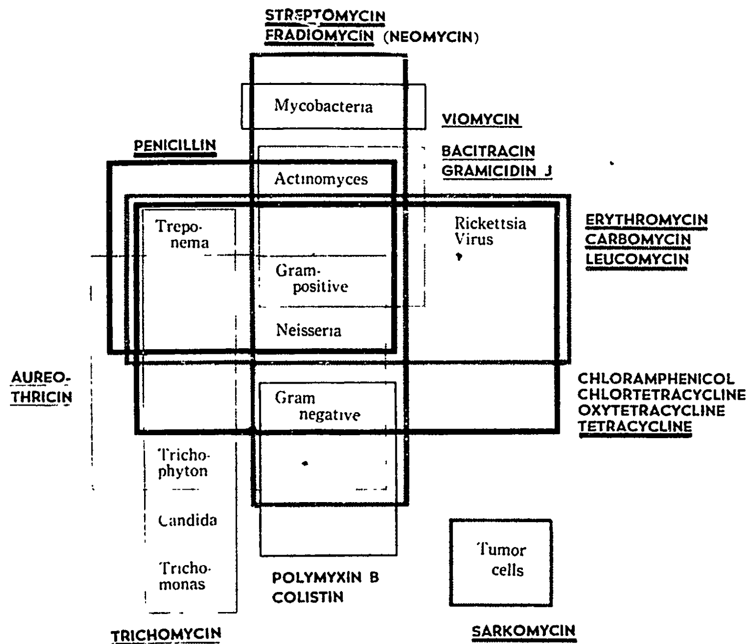
The newest phase in the field of antibiotics is to search for the substances with activities against neoplastic diseases including carcinoma, sarcoma and leucemia. Actinomycin provides some effects against such diseases, although it is toxic. Sarkomycin which was developed by Dr. Umezawa and tested clinicaly by Dr Ishiyama of Telecommunication Hospital is attracting world's attention in the respects that it can be administered to some malignant tumors without side reaction Recently, some other anti-cancer substances were reported; carzinophyllin by Dr Hata, and carzinomycin by Dr Hosoya.

How remarkably the public health in Japan was improved in consequence of the development of antibiotics will be evidently understood from the fact that average span of human life prolonged 15 years during past 10 years. Future project of antibiotic researches will be to search for new antibiotics effective against infections uncurable by present available ones and to devise countermoves for preventing serious side reaction such as anaphylaxis though it is infrequent The development of antibiotic application in veterinary, fisheries and agriculture will be expected in the future

(1 Jul., 1955. Y Yagisawa)



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Antibiotic Preparations in Japan and U.S.A.

	Organisms effected								Antibiotic preparations		Reference
	Spirochaeta	Gram-positive bacteria	Gram-negative bacteria	Mycobacterium tuberculosis	Rickettsia virus	Fungi	Protozoa Trichomonas	Malignant tumor	Japan	U. S. A	
I	+++	+++	−	−	−	−	−	−	Penicillin	Penicillin	
II	−	−	++	+++	−	−	−	−	Streptomycin	Streptomycin	
III	−	−	++	+	−	−	−	−	Fradiomycin (Neomycin)	Neomycin	topically, sometimes orally
IV	+	++	+++	+++	−	−	−	−	Chloramphenicol Chlortetracycline Oxytetracycline Tetracycline	Chloramphenicol Chlortetracycline Oxytetracyclin Tetracycline	
V	++	++	−	−	++	−	−	−	Erythromycin Carbomycin Leucomycin	Erythromycin Carbomycin	
VI	−	−	−	++	−	−	−	−	Viomycin	Viomycin	intramuscularly only
VII	−	−	++	−	−	−	−	−	Polymyxin B Colistin	Polymyxin B	
VIII	−	+++	−	−	−	−	−	−	Bacitracin Gramicidin	Bacitracin Tyrothricin	topically, sometimes orally
IX	+	−	−	−	−	++	+++	−	Trichomycin	Nystatin	topically, sometimes orally
X	−	−	−	−	−	++	−	−	Aureothricin		topically only
XI	−	−	−	−	−	−	−	++	Sarkomycin		intramuscularly only

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Čs. epidemiologie,
mikrobiologie, imunologie
V - 4 - 1956

Mikrobiologický ústav KÚNZ, Brno, přednosta prof. Dr V. Tomášek

EXPERIMENTÁLNÍ PATHOGENITA VIRUSU KLÍŠŤOVÉ ENCEFALITIDY PRO SSAJÍCÍ BÍLOU KRYSU*)

MUDr JAROSLAV PEŠEK za technické spolupráce VĚRY DOLEŽELOVÉ

Ssající bílá krysa je na rozdíl od dospělé značně vnímavá na infekci virusem klíšťové encefalitidy. První sdělení o tom u nás učinili Bárdoš a spolupracovníci (1954), kteří zjistili, že třídenní ssající bílé krysy hynou pátý den po i. c. podání nově isolovaného virusu klíšťové encefalitidy. Protože v literatuře nacházíme poměrně málo údajů o experimetální pathogenitě encefalitických virusů pro ssající bílou krysu, pokusili jsme se je doplnit u těch kmenů, s nimiž pracujeme. Koncem minulého roku jsme podali o našich výsledcích předběžnou zprávu (Pešek a Dluhoš 1954), z níž vyplývá, že některé encefalitické virusy se množí po naočkování do mozku dvoudenních ssajících bílých krys stejně dobře, nebo dokonce lépe, než v mozku bílých myšek 15 až 20 g těžkých.

Během dospívání klesá u bílé krysy nejen schopnost pomnožovat virus klíšťové encefalitidy v mozkové tkáni, nýbrž také vnímavost na infekci. Zvířata stará v den očkování 2 až 5 dnů hynula do týdne, zvířata stará 6 až 10 dnů do deseti dnů po očkování. Z krys starých v den očkování 12 dnů neuhynula do 21 dnů žádná, přestože některé z nich přechodně onemocněly.

Na základě těchto výsledků jsme vyslovili názor, že ssající bílá krysa je velmi cenným laboratorním zvířetem při studiu encefalitických virusů. Také Bárdoš již v květnu 1954 referoval o možnosti užití ssajících bílých krys při extraneurálních neutralisačních pokusech s virusem koňské encefalomyelitidy a některých jiných laboratorních pracích. Sami jsme se přesvědčili, že mozku ssajících bílých krys lze použít s výhodou při výrobě komplement-fixačních antigenů, vakcin, a že bílá krysa nám může dobře posloužit při výrobě imunních ser proti encefalitickým virusům.

Protože postupná ztráta vnímavosti během dospívání nám umožňuje volit k pokusům zvíře libovolně vnímavé, což by mohlo nabýt velkého významu při studiu experimentální therapie a podobných problémů, pokusili jsme se tento zjev blíže osvětlit pokusně. Jsme si vědomi, že výsledky předložených pokusů zdaleka nevyčerpávají celou problematiku tohoto velmi komplikovaného zjevu, že však nám umožňují některé dílčí závěry, které by mohly mít praktický význam.

METODIKA

1 Virus. Při pokusech jsme užili kmene Hy, který byl isolován naší laboratoří během epidemie klíšťové encefalitidy na Brněnsku roku 1953. Virus je udržován pasážováním na bílých myškách. Před pokusem jsme virus naočkovali dvoudenním bílým krysám, které jsme za čtyři dny usmrtili vykrvácením. Ze získaných mozků jsme připravili 10% suspensi ve fysiologickém roztoku, kterou jsme lehce odstředili (5 min. při 2000 obr./min.) a tekutinu nad sedimentem jsme užili jako inokulum.

2. Pokusná zvířata. Bylo užito 110 ssajících bílých krys starých 11 až 36 dnů, které jsme získávali vlastním chovem z matek kmene Wistar, dodávaných družstvem »Zverex«. Ve stáří 21 dnů jsme mláďata odstavovali. Pokud byla některá očkována v nižším věku, byla ponechána u matek do 21 dnů. Očkování jsme prováděli bez narkosy i. c. po 0,05 ml 10% suspense. Po naočkování byla zvířata sledována dvakrát denně a zaznamenávány příznaky onemocnění nebo případná uhynutí. V intervalech 7, 14, 24, 35 a 52 dnů byla vždy část zvířat v jednotlivých věkových skupinách usmrcena za i. p. chloralhydratové narkosy

*) Předneseno na I. celostátní urologické konferenci ve Smolenicích v říjnu 1955.

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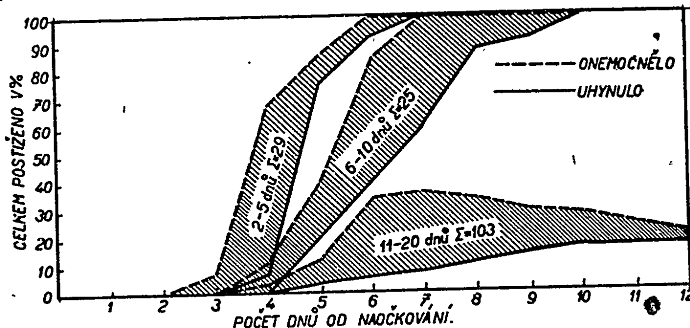
vykrvácením z a. axillaris. Krev byla zachycována a získané serum uchováno až do vyšetření zmražené v lednici při —20° C.

3. Serologická vyšetření. Po získání všech vzorků jsme vyšetřili sera na přítomnost komplement-fixačních a neutralisačních protilátek.

a) Vazba komplementu. Antigeny pro komplement-fixační reakci jsme připravovali podle Casalse a Palaciose (1941) a Casalse (1947) s tou změnou, že poslední odstředění jsme prováděli při 9000 obr./min. místo 3500 nebo 7000, jak je uvedeno v původních předpisech. Antigeny jsme konservovali merthiolatem 1:10.000 a uchovávali v lednici při 4° C, nikdy déle než tři měsíce a používali jsme je nezředěné. Reakci jsme prováděli v malých objemech (0,1 sera, 0,1 antigenu a 0,2 ml komplementu = 2 haemol. jed.). Sera byla těsně před reakcí inaktivována při zředění 1:2 zahřátím na 60° C po 20 min. Vazbu



Graf 1. Nemocnost a úmrtnost různě starých bílých krys po i. c. naočkování virusu klíšťové meningoencefalitidy.

jsme prováděli v lednici při 4° C po dobu 18 hodin a těsně před přidáním 0,2 ml 2% hypersensibilisovaných krvinek (Kabelík 1939) jsme reakce přenesli na 10 minut do vodní lázně 37° C.

b) Neutralisace. Neutralisační reakce jsme prováděli na bílých myškách váhy 15 až 20 g. Pro jistý nedostatek pokusných zvířat jsme byli nuceni vyšetřovat směsi ser (průměrně od pěti zvířat) získaných ve stejných časových intervalech od zvířat stejných věkových skupin. Sera byla smíchána stejným dílem a inaktivována při 56° C po 30 min. K 20% suspensi infikovaných myších mozků v 10% inaktivovaném morčecím seru, která byla dále logaritmicky zředěna 10% inaktivovaným morčecím serem, jsme přidávali stejný díl vyšetřované směsi ser. Po inkubaci 30 min. v pokojové teplotě jsme směs virusu a sera dále uchovávali v ledové lázni. Každým zředěním bylo očkováno 6 myšek i. c. po 0,03 ml. Naočkované myšky jsem sledovali 10 dnů a neutralisační index jsme vypočetli odečtením log LD_{50} zjištěného za přítomnosti vyšetřovaného sera od log LD_{50} zjištěného v kontrole s normálním krysím serem.

4. Isolace virusu z mozků infikovaných krys jsme prováděli po usmrcení zvířat důkladným vykrvácením v i. p. chloralhydrátové narkose. Ze sterilně vyjmutých mozků jsme připravili 10% suspensi ve fysiologickém roztoku, kterou jsme po lehkém odstředění očkovali v etherové narkose i. c. v množství 0,03 ml vždy šesti bílým myškám. Naočko-očkovali v etherové narkose i. c. v množství 0,03 ml vždy šesti bílým myškám. Naočkovaná zvířata jsme sledovali po tři týdny. Když se u některého z pokusných zvířat objevily příznaky onemocnění, bylo zvíře usmrceno a z mozku založena druhá pasáž, které bylo užito k přípravě antigenu podle Casalse. Za pomoci standardních laboratorních ser byla komplement-fixační reakcí prokazována identita virusu isolovaného s virusem původně naočkovaným.

5. Pokus o průkaz inhibitorů v mozku dospělé krysy jsme prováděli takto: 20% suspensi infekčního myšího mozku v 10% inaktivovaném morčecím seru jsme logaritmicky zředili 10% inaktivovaným morčecím serem a jednotlivá zředění jsme smísili aa:

1. s 10% inaktivovaným morčecím serem,
2. s 20% suspensí mozku normální dvoudenní krysy v 10% inaktivovaném morčecím seru a

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3. s 20% suspensí mozku normální dospělé krysy s 10% inaktivovaným morčecím serem.

Směsi jsme inkubovali 30 minut v pokojové teplotě a jednolivými zředěními očkovali vždy 6 myšek. Podle Reeda a Muenche (1938) jsme vypočetli pro jednotlivé případy hodnoty LD_{50} a methodou podle Pizziho (1950) standardní chybu těchto hodnot.

VÝSLEDKY

1. Nemocnost a úmrtnost různě starých ssajících bílých krys po i. c. naočkování virusu klíšťové encefalitidy (kmen Hy).

POČET DNŮ OD OČKOVÁNÍ	ISOLAČNÍ POKUS	PRŮKAZ VIRUSU KFR.	TITR PROTILÁTEK KF	TITR PROTILÁTEK NEUTR
24	7 7 8 9	+	1:64	107
24	7 8 9	+	1:32	
35	8 12	+	1:16	
35			1:16	
52	9	+	1:8	
52			1:128	

Graf 2. Isolace virusu z mozků krys očkovaných i. c. ve věku 11 dnů.

Vysvětlivky pro graf 2. — 2. sloupec: Vyšrafovaný čtverec = myš onemocněla, číslo pod ním uvedené znamená dni od očkování. — 5. sloupec: Neutralisační protilátky vyšetřeny ve směsi (aa) ser prvních tří zvířat.

Výsledky jsou společně s dříve získanými daty pro zvířata stará 2 až 10 dnů (Pešek a Dluhoš 1954) shrnuty v grafu 1. Ze zvířat starých v den očkování 21 až 36 dnů neuhynulo ani neonemocnělo během pozorovací doby (52 dnů) žádné. Ve skupině od 11 do 20 dnů jsme pozorovali jak zřetelné příznaky onemocnění, tak i případy hynutí. Zvířata stará v den očkování 11 až 13 dnů onemocněla všechna, u ostatních ve skupině do 20 dnů bylo pozorováno méně onemocnění, a to v nepřímé závislosti na věku. Zvířata starší se uzdravovala rychle, kdežto u mladších byl pozorován zdlouhavý průběh a zvířata jevila i po několika týdnech stále chorobné příznaky, hlavně zvýšenou dráždivost. Proto jsme se u zvířat očkovaných ve stáří 11 dnů pokusili o zjištění, jak dlouho přetrvává virus v organismu.

Výsledky šesti isolačních pokusů provedených za 24, 35 a 52 dnů po i. c. očkování jsou v grafu 2. Považujeme je za průkaz přetrvávání virusu v mozku. V době, kdy byl virus z mozků isolován, měla již všechna zvířata vytvořeny komplement-fixační protilátky a také neutralisační protilátky byly nejméně u jednoho zvířete přítomny. Protože u virusových nákaz viremie končí pravidelně s objevením se protilátek v krvi, předpokládáme, že isolovaný virus pocházel z buněk mozkové tkáně.

I když celý pokus byl proveden způsobem, který neodpovídá podmínkám přirozené infekce, ke které dochází výhradně cestou extraneurální, mohlo by zjištění, že virus tak dlouho přetrvává v organismu infikovaného zvířete, mít praktický význam epizootologický, kdyby se potvrdilo také u některých divoce žijících zví-

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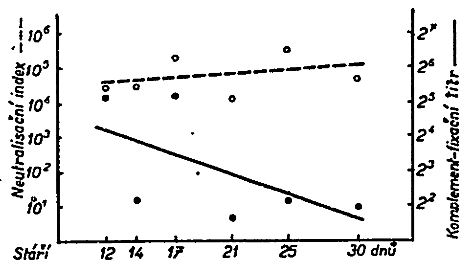
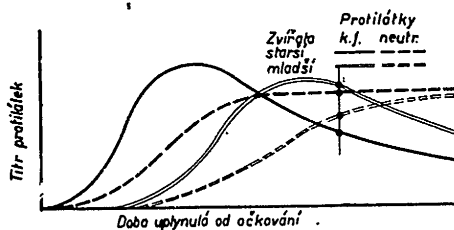
řat. Mladá zvířata, která jsou dostatečně odolná, aby infekci nepodlehla, a těch je v jarních měsících, kdy se virus v ohnisku počíná manifestovat, hojně, mohla by být dlouhou dobu nositeli virusu, a tak pomáhat jeho udržování a šíření.

2. Protilátková odpověď různě starých bílých krys po i. c. očkování virusem klíšťové encefalitidy.

U různě starých zvířat, která přežila pokus popsaný v předchozím sdělení (Pešek a Dluhoš 1954), jsme po 7 týdnech vyšetřili sera komplement fixační a neutralisační reakcí. Výsledky jsou uvedeny v první polovině grafu 3a. U mladších zvířat jsme zjistili po sedmi týdnech poměrně vysoké titry protilátek kom-



Graf 3a. Hladina protilátek krys očkovaných v různém stáří za 7 týdnů po očkování.



Graf 3b. Předpokládaný průběh imunisace různě starých krys.

plement-fixačních a nižší titry protilátek neutralisačních, kdežto u zvířat starších tomu bylo naopak. Z tohoto jednorázového vyšetření ser jsme sestrojili pravděpodobný průběh imunisace různě starých zvířat, jak to ukazuje druhá část grafu 3b.

Téměř současně jsme našli Overmanovo (1954) sdělení o protilátkové odpovědi ssajících bílých myšek po očkování parotitickou vakcinou. Protože jeho údaje byly v souhlasu s naší domněnkou, pokusili jsme se podrobněji sledovat průběh imunisace různě starých bílých krys po jediné i. c. injekci živého virusu klíšťové encefalitidy (kmen Hy).

Při hodnocení výsledků jsme zvířata rozdělili do dvou věkových skupin. První zahrnovala krysy staré v den očkování 11 až 20 dnů, mezi nimiž jsme pozorovali jak onemocnění, tak i hynutí. Ve druhé skupině byla zvířata stará v den očkování 21 až 36 dnů, která všechna zůstala po naočkování zdravá. Výsledky jsou v tabulce 1.

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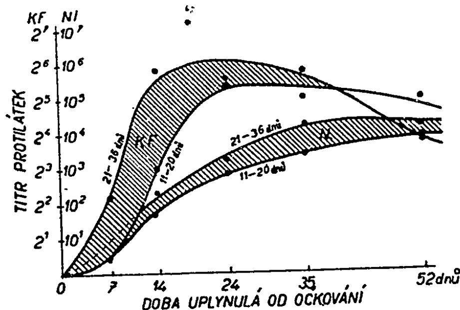
Tabulka 1. Průměrné hodnoty titrů KF a neutralisačních protilátek.

U bílých krys starých v den očkování		Za dnů				
		7	14	24	35	52
A) KF protilátky (rec. hod. titru)	11—20 dnů	$2^{0,41}$	$2^{3,0}$	$2^{5,32}$	$2^{5,0}$	$2^{4,93}$
	21—36 dnů	$2^{2,17}$	$2^{5,8}$	$2^{5,58}$	$2^{5,75}$	$2^{5,64}$
B) Neutralisační index (log)	11—20 dnů		1,70	2,82	3,37	3,81
	21—36 dnů		2,38	3,22	4,22	4,13

Graf 4, ve kterém jsou získané výsledky znázorněny křivkami, ukazuje, že dynamika imunisace odpovídá dříve vysloveným předpokladům. U starších zvířat pozorujeme rychlejší tvorbu jak komplement-fixačních, tak neutralisačních protilátek.

Přestože lze vidět určitou závislost odolnosti různě starých bílých krys na schopnosti tvořit rychleji protilátky, netvrdíme, že právě tento zjev je příčinou rozdílné vnímavosti různě starých zvířat, a to i tehdy, když Overman (1954) na základě podobných pokusů doplněných pasivní imunisací uzavírá, že právě vývoj imunitních mechanismů během dospívání má významnou roli při změnách vnímavosti živočichů.



Graf 4. Dynamika tvorby protilátek proti virusu klíšťové encefalitidy u ssajících bílých krys.

Dříve než bude možné o tom konečně rozhodnout, bude zapotřebí vyloučit všechny ostatní vlivy, jak je uvádí Sigel (1952), a teprve potom určit, zda jde o zjev, který má jedinou příčinu, nebo zda se na něm neuplatňuje celý komplex příčin.

Proto jsme se také v následujícím pokuse snažili o řešení dalšího dílčího problému.

3. Pokus o průkaz inhibitorů v mozku dospělé krysy.

Různá vnímavost mladých a dospělých pokusných zvířat se někdy vysvětluje přítomností inhibičních látek v seru nebo tkáních dospělých zvířat. Sigel (1952) cituje práci Casalse a Olitského, kteří zjistili, že lipidy obsažené v seru inhibují některé neurotropní virusy a sdělení Folchovo, který zjistil, že cerebrosidy a proteolipidy nejsou obsaženy v mozku novorozené myšky, a že se objevují teprve za 7 až 10 dnů po narození. Na základě těchto údajů jsme chtěli vyzkoušet, zda se tyto okolnosti neuplatňují také v našem případě a řešili jsme to titrací virusu klíšťové encefalitidy za přítomnosti mozkové tkáně ssající a dospělé krysy.

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Tabulka 2. Pokus o průkaz inhibitorů v mozku dospělé krysy.

	LD$_{50}$ (neg. log)
1. Myší mozek + morčecí serum	8,83 ± 0,39
2. Myší mozek + mozek dvoudenní krysy	8,75 ± 0,35
3. Myší mozek + mozek dospělé krysy	9,16 ± 0,38

Výsledky jsou v tabulce 2. a ukazují na to, že v mozku dospělé krysy nejsou přítomny inhibitory, které by snižovaly infekční titr virusu klíšťové encefalitidy.

DISKUSE

Předložené výsledky nepovažujeme za konečné řešení problému rozdílné vnímavosti různě starých bílých krys na infekci virusem klíšťové encefalitidy. V tom směru bude zapotřebí vyřešit řadu dalších dílčích otázek, jak jsme se to pokusili nadhodit. Především problém persistence virusu v organismu infikovaných zvířat bude zapotřebí dořešit šířeji založenými pokusy. Získali jsme však řadu údajů o průběhu experimentální infekce, což skýtá základnu k užití ssajících krys při laboratorní práci s některými encefalitickými virusy.

SOUHRN

Byl sledován průběh experimentální infekce různě starých bílých krys virusem klíšťové encefalitidy. Ze zvířat starých 21 až 36 dnů v den očkování neuhynulo nebo neonemocnělo žádné. Zvířata stará 11 až 13 dnů onemocněla všechna a některá uhynula. U ostatních zvířat ve skupině do 20 dnů bylo pozorováno stále méně onemocnění.

Jedenáctidenní krysy přechovávají po i. c. injekci v mozku virus až 52 dnů.

Bylo zjištěno, že mladší zvířata tvoří protilátky pomaleji než zvířata starší.

Byl proveden pokus o průkaz inhibitorů v mozku dospělé krysy titrací virusu za přítomnosti tkáně dospělé a dvoudenní krysy. V hodnotách nebylo významného rozdílu.

Zdůrazňuje se vhodnost ssajících krys pro práci s encefalitickými virusy.

ВЫВОДЫ

Експериментальный патогенитет вирусов клещевого енцефалита для сасунков белых крыс

Наблюдался ход експериментальной инфекции белых крыс разного возраста вирусом клещевого енцефалита. Из привитых животных в возрасте от 21 до 36 дней в день прививки не заболело или не погибло ни одно. Животные в возрасте 11—13 дней заболели все и некоторые погибли. У остальных животных в группе до 20 дней возраста заболевание наблюдалось реже.

Крысы в возрасте 11 дней после интрацеребральной прививки сохраняют в мозгу вирус до 52 дней.

Установлено, что молодые животные вырабатывают антитела медленее чем старшие животные.

Произведен опыт чтобы проверить присутствие ингибиторов в мозгу взрослой крысы методом титрации вируса в присутствии ткани взрослой и двухсуточной крысы. В результатах не было значительной разницы. Авторы обращают внимание на возможность употребления сосунков крыс для работы с вирусом енцефалита.

SUMMARY

Experimental Pathogenity of the Tick Encephalitis Virus for Suckling Albino Rats

The course of experimental infection with the virus of tick encephalitis in albino rats of various age was observed. None of the animals inoculated between 21st and 36 th day of life grew ill or died. All of the 11—13 days old rats showed symptoms of the disease and some of them died. A descendant morbidity rate could be observed in the remaining group of 14—20 days old rats.

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The virus has been found to persist for 52 days in the brain of 11 days old rats after intracerebral inoculation.

It has been found that antibody formation is slower in younger animals than in the older ones.

We have attempted to prove the presence of inhibitors in the brain of grown-up rats. For this purpose we carried out virus titration in the presence of the tissues of grown-up and two days old rats without significant difference in the results.

The possibility to use suckling rats for the work with encephalitic viruses is stressed.

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SEPARATUM

ACTA MICROBIOLOGICA
ACADEMIAE SCIENTIARUM HUNGARICAE

TOMUS III FASCICULUS 4

M. PINTÉR and E. ÁBRAHÁM

GROWTH OF MM VIRUS IN HUMAN EMBRYONIC TISSUE

1956

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GROWTH OF MM VIRUS IN HUMAN EMBRYONIC TISSUE

By

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(Received, January 24, 1956)

Since the virus strains belonging to the encephalomyocarditis (EMC) group, such as the Col SK, MM, EMC, Mengo, strains had been discovered, the biological properties of this virus group have been dealt with in numerous reports. Its relation to poliomyelitis virus was a particularly controversial issue, so long as it has not been established that no serological relationship existed between the two groups of virus [1, 2]. All its strains having been isolated from animals, there was doubt about the pathogenicity of EMC virus for man. At first, only serological evidence had suggested that they might cause disease in man [3]; more recently, however, the virus was isolated also from human cases [4, 5, 6]. According to present knowledge, in grave cases there are encephalomyelitis and/or serous meningitis, but abortive and asymptomatic forms may also occur.

In general, the viruses of the EMC group grow readily in the chicken embryo and tissue cultures alike. The pertaining reports deal mainly with the cultivation of the Col SK and MM viruses, but it can be assumed that the other members of the group do not greatly differ in cultural properties. SANDERS and JUNGEBLUT [7] studied the growth of MM virus in different Maitland-type cultures of chicken embryo and mouse embryo tissues and found the virus passable in cultures prepared from embryonic chicken tissue, embryonic mouse brain and lung. CHAMBERS et al. [8] showed that the MM virus grew slowly and slightly in suspended mouse and human testicle tissue cultures but could not systematically passage the virus. More recently, SMITH and EVANS [9] have used monkey testicle, and FABIYI [10] mouse testicle roller tube cultures for cultivating MM virus and found that the virus grew abundantly in these cultures. The multiplication of virus in both tissues is accompanied by a degeneration of fibroblasts.

In the following, we shall report on the growth and cell pathogenicity of MM virus cultured in human embryonic tissue.

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Materials and methods

The strain of MM virus used in the experiments has been obtained from DR. W. RHODE (Jena), in the form of infected mouse brain. The strain was passaged and found to have a mouse infectivity titre varying from 10^{-6} to 10^{-8}. The animals infected died in 2 to 5 days, with the characteristic symptoms of encephalomyelitis.

Multiplication of virus was studied in cultures prepared from skin and muscle from 2 to 4-month-old human embryos. The pieces of tissue were cultured at 36 °C, in a thin layer of hen plasma, with the stationary tube culture technique. The nutrient fluid, of which 2 ml was added to each tube, consisted of chicken embryo extract 10 per cent ; horse serum 5 per cent ; HANKS' solution, 85 per cent ; with 50 μg of penicillin and 50 μg of streptomycin per ml. Infection was carried out with 0,2 ml of inoculum on the 3rd or 4th day of culturing. Before adding the new nutrient fluid, the tissues were allowed to remain in contact with the inoculated material for 30 minutes.

Tenfold-diluted viral material and serum of 1 : 10 end dilution were used in the neutralisation tests. The serum-virus mixture was kept at room temperature for 1 hour and after that 0,2 ml of it were transferred into each tube.

The specific antiserum to MM virus was produced in rabbits. Each rabbit was inoculated three times in intervals of one week, with 1,0 ml of a 10 per cent suspension of MM virus-infected mouse brain, by the intramuscular route. 10 days following the last inoculation the animals were bled. The sera obtained had a neutralisation index of 10 000, as determined in the mouse.

Results

The starting material was a mouse brain suspension of $10^{-6.5}$ titre, tenfold dilutions of which were used for infecting the human embryonic tissue cultures. A 10^{-1} dilution of this viral material 18 hours after infection produced a clearly visible degeneration of fibroblasts and after 48 hours no normal cells were visible in the culture. At a dilution of 10^{-7} the degeneration developed on the third day, while a 10^{-8} dilution produced no cell lesion. The degenerating cells were characteristically elongated and contained fine granules in the cytoplasm. Another conspicuous feature was that, even with advanced degeneration, the cells remained grouped in their original, radial order. These characteristics make it possible to distinguish to a certain extent the cytopathogenic changes produced by the virus from those brought about by the poliomyelitis virus.

Next, by passaging the virus in tissue cultures the 16th passage was reached. During passage we titrated the virus in tissue culture on 5 occasions and found the titre to vary between 10^{-4} and 10^{-6}. In two cases parallel titrations were carried out in the mouse ; the titres yielded were in satisfactory agreement with those obtained in the tissue culture. Fluid from the 7th passage was examined by the neutralisation test, in an effort to determine whether the cytopathogenic agent passaged in the tissue culture was identical with the original MM virus. The rabbit sera, which had proved specific in mouse experiments, neutralised up to 10 000 tissue culture LD_{50}. In view of this the virus passaged in the mouse may be considered identical with that passaged in tissue culture.

In the 9th passage the growth curve of the virus was established. Each of a number of tubes was infected with 1000 LD_{50} of virus and after 30 minutes the tubes were washed with Hanks' solution to remove virus not bound to cells.

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Nutrient fluid was then added and cultivation was continued in the usual manner. Meanwhile, at regular intervals the contents of 4 tubes were pooled and the fluids thus collected were stored at −20 °C. The pools were then titrated in tissue culture.

In Fig. 1 are shown the titres obtained at different points of time. After 6 hours the titre was as low as 10^{-1}, meaning that only part of the inoculated virus could be demonstrated. The rate of multiplication then increased rapidly, to reach the maximum titre of 10^{-5} in the 24th hour. Subsequently the titre

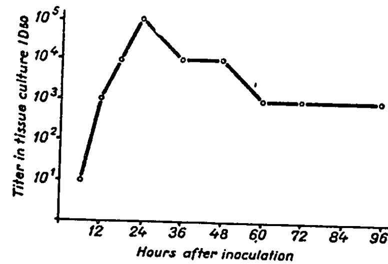

Fig. 1. Growth of MM virus in human embryonic tissue culture

slowly declined, but as late as after 96 hours a demonstrable quantity of virus was still considerable. As to the relation between multiplication rate and development of cytopathogenic action, the latter was found somewhat to lag behind the former, inasmuch as the complete degeneration of cells took as much as 48 to 64 hours.

Discussion

We have succeeded in growing MM virus, a member of the EMC group, in human embryonic tissue. In the only report on successful growth of MM virus in human tissue [8] no clear-cut evidence can be found as regards the relationship of the virus to human tissue. Our results indicate that in human embryonic tissue the MM virus multiplies at the same rate as in mouse or monkey tissue cultures. This agrees with the clinical observations, which all suggest that the MM virus is capable of growing in the human organism. VERLINDE and VAN TONGEREN [6] have contributed valuable evidence to this point when they recovered a virus strain belonging to the EMC-group from a case believed to be one of poliomyelitis, and from another showing the symptoms of encephalomyelitis.

Our method for growing the MM virus seems to be suitable for use in neutralisation tests on a larger scale. The method is less costly than the mouse

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test and is apparently more specific than the haemagglutination-inhibition test. In informative trials attempts have been made to use the method for the demonstration of antibody. Sera from 165 normal individuals were examined by the neutralisation test and the presence of neutralising antibodies could be shown even by repeated testing in 4 cases. These investigations still await corroboration by animal experiments and will therefore not be evaluated here. Should the positive sera possess a specific neutralising action, this will be interpreted as a proof of the occurrence of viruses belonging to the EMC group virus in Hungary.

Summary

The MM virus can be grown readily in fibroblast culture prepared from human embryonic skin. The virus reaches its maximum titre (10^{-5}) in 24 hours and causes meanwhile an initial degeneration of cells, resulting ultimately in total tissue destruction. The method described is suitable also for use in neutralisation tests.

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КУЛЬТИВИРОВАНИЕ ВИРУСА ММ В ЧЕЛОВЕЧЕСКИХ ЭМБРИОНАЛЬНЫХ ТКАНЯХ

М. ПИНТЕР и Э. АБРАХАМ

Резюме

Вирус ММ, относящийся к группе энцефаломиокардитов, хорошо размножается в культуре фибробластов из кожи и мускулатуры человеческого зародыша. В 24-ом часу после инокуляции титр вируса достигает максимального значения (10^{-5}). Одновременно с размножением вируса наблюдается дегенерация клеток, и процесс этот приводит к полной гибели клеток. Описанный способ культивирования пригоден для постановки реакций нейтрализации. С помощью реакции нейтрализации в сыворотках нескольких процентов здоровых лиц удалось обнаружить антитела, нейтрализующие вирус ММ.

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Aus dem Mikrobiologischen Institut der Universität
und des Klinischen Krankenhauses in Brünn
(Vorstand Prof. Dr. Václav Tomášek)

Ein Beitrag zur Serologie der Zeckenenzephalitis (Z. E.)

L. Jandásek und **J. Pešek**

(Eingegangen bei der Schriftleitung am 6. März 1956)

Zum indirekten Nachweis von Z. E. gibt es zur Zeit zwei praktisch anwendbare Reaktionen: Die Komplementbindungsreaktion (KFR) und den Seroneutralisationstest (SNT). Die erste von diesen Proben führen wir mit den nach Cassals und Palacios hergestellten Antigenen (1, 2) durch, die in letzter Zeit der Kritik vielmals unterworfen wurden. Unsere Mitteilung soll beweisen, wie sich diese Antigene in der Praxis und im Vergleich mit den Ergebnissen der SNT bewähren.

Die zweite Reaktion (SNT) wird wegen ihrer größeren Spezifizität und wegen der Möglichkeit der retrospektiven Diagnostik einer vor längerer Zeit durchgemachten Krankheit allgemein höher eingeschätzt. Ihre praktische Anwendbarkeit wird jedoch durch den hohen Preis der Versuchstiere, durch die zeitraubende Technik und endlich durch die Notwendigkeit einer gutgeführten Tierzucht vermindert. Um diese Nachteile zu beseitigen, haben wir die Möglichkeit der Verwendung von Bruteiern statt den üblich benützten Mäusen untersucht.

Die Versuche über die Verwendbarkeit von Eiern zum Nachweis der virusneutralisierenden Antikörper bei Neurovirosen sind aus den Arbeiten von Blattner und Cooke (3) für St. Louis Enz., von Koprowski und Lenette (4) für Venezuela-Enzephalitis, von Crawley (5) für die Pferde-Enzephalitis und von Imagawa et al. (6) für Hundestaupe bekannt. In der Praxis wurde jedoch diese Methode nur bei den Viren, die Erythrocyten agglutinieren, und bei den herpetischen Infektionen [Horsfall (7)] angewendet.

Die Vorteile, die sich aus der Anwendung von Eiern statt Mäusen ergeben, sind folgende:

1. Billigkeit. Man kann rechnen, daß der Preis eines Eies etwa ein Drittel des Preises einer weißen Maus beträgt. Bei den Eiern entfällt die Fütterung.

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2. Arbeitsersparnis. Abgesehen davon, daß die eigentliche Impfung der Eier viel bequemer ist, entfällt die Fütterung von Mäusen, die Reinigung der Käfige und die tägliche Kontrolle der geimpften Tiere.
3. Raumersparnis. Zehn SNT kann ein einziger Thermostat fassen, während für Mäuse ein ganzer Raum notwendig ist.
4. Es gibt keine Gefahr von Enzootien, die die Unverläßlichkeit der Proben verursachen oder die Liquidation der ganzen Zucht erzwingen können (Salmonella, Ektromelie).

Dagegen muß man mit folgenden Nachteilen bei Verwendung der Eier gegenüber der Verwendung von Mäusen rechnen.

1. Größere Möglichkeit der Kontamination. Dieser Umstand spielte am Anfang unserer Arbeit eine große Rolle. Die Blutproben wurden oft nicht steril entnommen, oder sie wurden während der Verarbeitung im Laboratorium kontaminiert. Diesen Nachteil haben wir praktisch durch Zugabe von Antibiotika, die von Embryonen auch in hoher Konzentration gut vertragen werden, und besonders durch Inaktivieren der Sera völlig beseitigt. 2. Größere durch das unspezifische Absterben verursachte Verluste. Dies ist ein ernster Nachteil bei der Verwendung der Eier. Die Embryonen sterben einerseits während der Inkubationszeit vor dem Versuch, andererseits bis zu 48 Stunden nach dem Impfen infolge eines Traumas oder einer Kontamination ab. Die erste Periode des Absterbens kann man gewissermaßen durch pünktliches Einhalten der optimalen Bedingungen im Brutofen beseitigen. Von größter Bedeutung ist dabei die Farm, welche die Eier liefert. Die Zahl der sterilen Eier (die man übrigens zur Verfertigung von bakteriologischen Böden benützen kann) und der Eier, die bis zum vierten Tage der Inkubationszeit unspezifisch absterben, betrug bei uns höchstens 25%, durchschnittlich 10%. Das unspezifische Absterben nach der Inokulation der Teste macht 11% aus. Es gelang uns nicht mehr diese Grenzzahl herabzusetzen. Bei den Mäusen macht das unspezifische Absterben nach der Inokulation 3% aus.

Bei der Arbeit mit Viren, die nicht die Blutkörperchen agglutinieren, kann man Eier nur dann benützen, wenn die Viren den Embryo töten oder sichtbare Änderungen auf der Chorioallantois verursachen. Die ersten Versuche mit den SNT führten wir mittels der Impfung auf Chorioallantois durch. Obzwar alle Autoren, die einige Stämme des Zeckenenzephalitisvirus in der ČSR isoliert haben [Galia et al. (8), Krejčí (18), Jandásek et al. (10), Bárdoš (11)] die Entstehung von sichtbaren Herden nach der Impfung auf die Chorioallantois beschrieben, kamen wir zur Ansicht, daß diese Methode für die Praxis nicht anwendbar ist. Abgesehen von der mühsamen Arbeitsprozedur, welche die Impfung auf die Chorioallantois verlangt, ist es vor allem die Unverläßlichkeit der sichtbaren Veränderungen, die diese Technik entwertet. Zweckmäßiger ist die Impfung in den Dottersack, benützt von

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Crawley (5, 12), für Pferdeenzephalitisvirus, von Koprowski und Cox (13) und Howitt (14) für Japanenzephalitisvirus, von Burnet (15) und Edward (16) für Louping-ill-virus. Diese Methode hat alle Voraussetzungen auch für die praktische Diagnostik der Zeckenenzephalitis, wie wir in einer früheren Arbeit bewiesen hatten (24). Der Embryo stirbt konstant bis zum achten Tage nach der Inokulation, ohne jede Adaptation des Virusstammes. Die Empfänglichkeit ist im Laufe des ganzen Jahres konstant. Die auf den Eiern festgestellten Virustiter stimmen mit denjenigen auf Mäusen überein.

Material und Methoden

I. Komplementbindungsreaktion

Das Antigen stellten wir nach Cassals und Palacios (1, 2) mit kleinen Veränderungen her. Die Mäuse entbluteten wir aus der A. axillaris, um möglichst vollkommene Entblutung des Gehirngewebes zu erreichen. Das letzte Zentrifugieren führten wir bei 9000 Umdrehungen/Minute anstatt 3500 resp. 7000 der zitierten Autoren durch. Zur Antigenherstellung verwendeten wir zwei Stämme, die beide in Mähren isoliert worden waren, und zwar Stamm Hypr (17) und Stamm Čs 4-B-12, isoliert von Krejčí (18). Die Antigene wurden unverdünnt verwendet. Dann wurden sie im Eisschrank bei 4° C nicht länger als drei Monate aufbewahrt.

Die untersuchten Sera wurden gleich nach dem Eintreffen ins Laboratorium abgesondert und bei −10° C aufbewahrt. Vor der Untersuchung, die spätestens während einer Woche zustande kommt, wurden sie mittels phys. Lösung 1 : 2 verdünnt und 20 Minuten bei 60° C inaktiviert. Die eigentliche Reaktion wurde in kleinem Volumen durchgeführt: 0,1 ml Serum, 0,1 ml Antigen und zwei Einheiten Komplement in 0,2 ml. Nach der Inkubation von 18 Stunden bei 4° C gaben wir die Eprouvetten 10 Minuten lang in ein Wasserbad 37° C, dann gaben wir 0,2 ml von 2% hypersensibilierten (19) Schaf-Erythrozyten zu. Bei allen Kranken wurde BWR durchgeführt.

II. Seroneutralisationstest

1. Die Eier

Wir benutzten Eier von weißen Leghorn aus einigen Farmen in der Umgebung von Brünn. Die Eier wurden auf übliche Weise behandelt und in einem Brutofen bei 37° C und 50–70% Feuchtigkeit in horizontaler Lage inkubiert. Sie wurden täglich umgelegt. Am vierten Tage fand die Kontrolle der Fertilität und der Lebensfähigkeit der Embryonen mittels Durchleuchtung statt.

Zur Impfung benutzte man stets sieben Tage alte Embryonen, die nach der Impfung in vertikaler Lage in einen bakteriologischen Thermostat bei 36° C gelegt wurden. Die Atmosphäre im Thermostat wurde mittels eines Gefäßes mit Wasser befeuchtet.

2. Die Virusstämme

Wir benutzten den in unserem Laboratorium im Jahre 1953 isolierten Stamm Hypr (17). Die virulente Suspension wurde folgendermaßen vorbereitet: Zehn Mäuse von 15 g Gewicht wurden mit dem Vorratstamm ins Gehirn geimpft. Die Tiere, die am vierten und fünften Tag Symptome von Enzephalitis auswiesen, wurden mittels Äther getötet und das Gehirn steril entnommen. Nach der bakteriologischen Kontrolle wurden die sterilen Gehirne in der phys. Lösung unter Zugabe von 10% des inaktivierten Meerschweinchenserums zerrieben. Dann wurden sie zehn Minuten bei 2500 Umdrehungen/Minute zentrifugiert, und die

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Suspension wurde in kleine Fläschchen mit Gummistöpseln zu 1 ml verteilt. Die auf diese Art hergestellte Suspension wurde in einen Eisschrank bei -10 bis --20° C gelegt, wo sie bei unveränderten Titer bis drei Monate erhalten blieb. Jede Charge wurde mittels der Methode von Reed und Muench (20) titriert

3. Die Mäuse

Wir benutzten Mäuse von 13 bis 20 g Gewicht, Stamm H., gefüttert mit Hafer und weißem Brot.

4 Die Durchführung der Reaktion

a) Die Herstellung der Mischungen von Sera mit dem Virus Wir inaktivierten das geprüfte Serum 30 Minuten bei 56° C. Dann pipettierten wir es zu 0,5 ml in die Eprouvetten. Das Virus verdünnten wir aus den Vorratsfläschchen mit dem Verdünnungsmittel von folgender Zusammensetzung Meerschweinchen-serum 2 ml, Penicillin 20000 E., Streptomycin 10 mg, phys. Lösung ad 20 ml. Die gewöhnlich benützten Verdünnungen des Virus waren für die unter-suchten Sera 10^{-3}, 10^{-4}, 10^{-5}, evtl. 10^{-6} Als das bekannte negative Serum diente uns das inaktivierte Meerschweinchenserum. Wir benutzten dafür das um eins bis zwei Logarithmen mehr verdünnte Virus Die Virusverdünnungen gaben wir zu den Sera ana partes. Dann inkubierten wir die Mischungen 30 Minuten bei 36 C.

b) Die Impfung der Mäuse geschah intrazerebral in Äthernarkose Das Inokulum betrug 0,03 ml Die geimpften Tiere wurden 10 Tage betrachtet Die bis zu 48 Stunden Abgestorbenen wurden als unspezifisch bewertet Mit jeder Ver-dünnung wurden fünf Mäuse geimpft

c) Die Impfung der Eier Sieben Tage alte Bruteier trepanierten wir mittels eines Stachels in der Mitte des Luftraumes. Zur Impfung verwendeten wir 35 mm lange Injektionsnadeln. Das Inokulum betrug 0,1 ml Den Stich führten wir in die Mitte des Eies, ohne Durchleuchtungskontrolle Mit jeder Verdünnung impften wir fünf Eier Dann inkubierten wir dieselben bei 36 C

d) Die Kontrolle der geimpften Eier fand mittels Durchleuchtung statt Die erste Kontrolle führten wir nach 48 Stunden nach der Impfung durch Dabei schlossen wir die toten Embryonen als Folge des Inokulationstraumas aus. Die zweite und endgültige Betrachtung des Testes führten wir acht Tage nach der Impfung durch Die toten Embryonen waren leicht an dem ver-wischten Bilde der Blutgefäße und an der Unbeweglichkeit zu erkennen

e) Das Berechnen des Neutralisationsindexes (N.I) Log DL 50 der negativen Kontrolle - -Log DL 50 des geprüften Serum Log N I Ein N.I von 50 und mehr wird als positiv bewertet

Ergebnisse

Mit KFR untersuchten wir während der letzten Jahre 2058 Sera von 1343 Personen, bei denen der Verdacht auf Z.E. ausgesprochen war Nur in 40 Fällen (1,94%) konnten die Ergebnisse nicht bewertet werden, da die Sera entweder mit negativen Antigen reagierten, oder eine Antikomplementärwirkung aufwiesen (Tabelle 1). Diese Eigen-schaft kommt in den Sommermonaten bei den mit der Post eingesand-ten Sera häufiger vor.

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Tabelle 1

Übersicht der Untersuchungen mittels KFR

Jahr	Anzahl der untersuchten		Davon waren	
	Proben	Personen	positiv	unbewertbar
1953	569	445	85	9
1954	756	393	138	14
1955	733	505	76	17
Gesamt	2058	1343	299	40 = 1,94%

Bei 299 Kranken gelang es uns die Diagnose von Z.E. mittels KFR festzustellen. Die Ergebnisse aus dem Jahre 1953 wurden an anderem Ort (25) mitgeteilt. Als positiv nehmen wir zur Zeit diejenige Reaktion an, die mindestens eine viermalige Steigerung des Titers während der Krankheit aufweist oder — falls zwei Blutproben nicht zur Verfügung stehen — einen Titer von 1 : 16 oder höher zeigt. Titer 1 : 8 bewerten wir als verdächtig, wenn nur die KFR zur Verfügung steht. Eine Reihe der positiven SNT bei diesen KFR-Titers zeigt, daß diese Ansicht berechtigt ist. Slonim (23) der die benzenextrahierten Antigene benützt, beurteilt schon die in der Verdünnung 1 : 4 reagierenden Sera als positiv. Die Zeiteinteilung der positiven Fälle stimmt sehr gut mit dem Saisoncharakter der Z.E. überein (Tabelle 2).

Tabelle 2

Übersicht der serologisch festgestellten Fälle von Z.E. nach den Monaten dem Beginn der Krankheit nach

Jahr	I	II	III.	IV	V	VI	VII.	VIII	IX	X	XI	XII
1953	0	0	0	0	0	13	34	23	5	10	0	0
1954	0	0	0	0	8	25	71	27	3	2	2	0
1955	0	0	0	0	1	12	23	32	5	3	0	0

Tabelle 3 und 4 zeigt den Vergleich der KFR mit den SNT. Aus diesem Vergleich gehen folgende Ergebnisse hervor:

1. SNT sowohl auf Eiern als auf Mäusen ist bei einer positiven KFR niemals negativ.
2. Die Sera der Personen, die vor einer längeren Zeit Z.E. durchgemacht hatten (Tabelle 3) erweisen oft einen positiven SNT nicht nur auf den Eiern sondern auch auf Mäusen, obwohl die KFR schon negativ ist.
3. Die SNT auf Eiern stimmen mit denen auf Mäusen in Hinsicht der Negativität und Positivität qualitativ überein, wobei jedoch quan-

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titative Unterschiede zum Vorschein treten. Der arithmetische Durchschnitt der konkret ausgedrückten Indexe beträgt bei den Eiern 1339, bei den Mäusen 2268. Diese Differenz ist teils durch höhere Titers der Serum-Virus-Mischungen bei den Eiern (3,9 bei den Eiern gegen 3,6 bei Mäusen)[1]), teils durch niedrigere Titers der negativen Kontrollen (6,1 bei Eiern, gegen 6,7 bei Mäusen) verursacht.

4. In den drei Fällen (5 27%) war SNT auf den Mäusen positiv, auf den Eiern negativ. In diesen Fällen betrug die KFR in dem gleichzeitig untersuchten Serum maximal 1 8.

Man kann also den Schluß ziehen, daß die sieben Tage alten in den Dottersack geimpften Bruteier, ein entsprechendes Objekt für die praktische Durchführung der SNT zwecks der Diagnose der Z.E. bieten. Die geringe Zahl der bei dieser Methode wahrscheinlich entgehenden positiven Fälle, wird durch eine wesentliche Steigerung der Finanz- Arbeits- und Raumkapazität des Laboratoriums kompensiert.

Die in unserer Mitteilung angeführte Technik der KFR hat sich schon in der Praxis bewährt (9). Wir befassen uns damit genau, weil eine Reihe von Arbeitern die ursprünglichen Wasserantigene im Vergleich mit Benzen- oder Chloroformextrakten für minderwertig halten (21, 24). Unsere Erfahrungen beweisen jedoch, daß die alten Casals- und Palacois-Antigene sehr zuverlässig für die diagnostische Praxis sind, besonders wenn man einige Verbesserungen, wie das Zentrifugieren bei höheren Umdrehungen, ausnützt. Entscheidend ist weiter das Benützen von frischen, höchstens drei Monate alten Antigenen. Wir stimmen völlig mit Smadel (22) überein, der in seiner Kritik der auf verschiedene Weise hergestellten Antigene sagt: „.... each (method) is satisfactory in the hands of those, familiar with it".

Man kann wohl die KFR als Beweis einer vor länger Zeit verlaufenen Krankheit nicht benützen, da die K.F.-Antikörper verhältnismäßig rasch aus dem Blute verschwinden (23, 25), wie das die Verfolgung der Dynamik von K.F.-Antikörper während der Erkrankung und der Konvaleszenz zeigt.

Zusammenfassung

Mittels Komplemetbindungsreaktion wurden 2058 Sera von den auf Zeckenenzephalitis verdächtigen Kranken untersucht. Die dabei benützten Wasserantigene nach Casals und Palacois bewährten sich als in der Praxis gut anwendbar. Nur 1,94% von Reaktionen waren wegen der Antikomplementärtwirkung oder unspezifischer Bindung der Sera unbewertbar. Außer der epidemischen Saison beobachtete man keine positiven Reaktionen.

[1]) Die in Klammern eingeführten Zahlen sind arithmetische Durchschnitte von den Logarithmen DL 50 mit Sera Nr 13, 20, 1448, 1468, 1474, 1611, 1634, 2156, 2157 und 2162 gemischten Virus, wie auch von ihren Kontrollen

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Tabelle 3

Prot Nr	Komplementbindungsreaktion: Tag[1]	Čs 4-B-12[2]	Hypr[3]	Seroneutr Test: Tag	Maus	Ei
1290	21	4	2			
	32	16	16			
	48	4	4			
	81	4	4			
	97	2	2			
	378	0	0	378	600	>1450
1609	700	0	0	700	165	1000
1611	35	64	—[4]			
	259	4	4			
	600	0	0	600	100	320
1614	506	0	0	506	0	10
1615	92	8	-			
	269	2	–			
	626	2	2	626	330	>430
1616	9	16	—			
	129	128	—			
	600	4	2	600	>150	250
1617	17	32	-			
	139	16	-			
	253	0	—			
	614	2	0	614	5610	3410
1618	17	16	-			
	611	2	0	611	2100	2200
1619	16	32	-			
	37	32	—			
	600	4	2	600	3500	2150
1625	3	0	—			
	564	0	0	564	8100	10000
1626	35	32	-			
	40	64	-			
	94	64	—			
	610	4	4	610	6300	2100
1627	37	8				
	43	32	—			
	577	4	2	577	10000	2250
1633	19	4	0			
	60	16				
	120	32	-			
	610	-		640	63	>200
1646	11	0	0			
	319	—	—	319	0	0
1664	21	0	0			
	26	0	0			
	40	0	0			
	367	0	0	367	<500	<500
1665	7	0	0			
	365	0	0	365	<500	<500
1666	365	0	0	365	<500	<500
1670	44	8	2			
	61	8	4			
	380	0	0	380	7,6	0

[1] Tage nach Beginn der Krankheit.
[2] Rezipr. Wert der Serumverd. mit Stamm Čs 4-B-12.
[3] Rezipr. Wert der Serumverdünnung mit Stamm Hypr.
[4] Nicht gemacht oder unbewertbar.

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Tabelle 4

Prot. Nr	Komplementbindungsreaktion	Seroneutr Test Maus	Ei
13	*1* *3* 2 2 8 8	*3* 160	6
20	*9* 4 4	*9* 470	22
25	*28* *56* *80* 4 4 16 8 16 8	*28* 135	145
137	*11* *24* *42* 4 4 64 64 64 64	*42* > 6300 —	1430
138	*?* 16 8	*?* 1000	330
1262	*10* *18* *33* *57* *107* *326* 8 4 32 16 8 8 8 4 4 8 0 0	*107* 187	540
1271	*18* *25* *73* 2 4 2 0 8 8	*73* 10000	615
1291	*6* *14* *42* *58* 4 4 4 4 8 8 8 8	*58* > 320	780
1292	*6* *13* *97* 2 2 0 0 0 0	*97* 3,2	6,4
1293	*22* *44* 16 16 16 16	*44* > 320	336
1295	*15* *22* *26* *31* *50* *80* 2 0 8 0 8 8 4 2 4 4 8 4	*80* 5000	680
1447	*18* *31* *45* 4 8 8 8 8	*45* 690	1350
1453	*20* 0 0	*20* < 1	1,65
1463	*46* *60* *98* 16 16 32 32 2 2	*98* 1200	100
1466	*14* *21* *62* 8 8 16 16 4 4	*62* 950	100
1468	*27* 8 8	*27* 1100	235
1472	*4* *17* *34* 4 4 8 8 32 32	*17* 660	850
1473	*17* *34* *55* *76* *93* 0 0 4 4 8 8 16 16 32 16	*76* 680	1100
1474	*17* *22* *35* *57* *78* 16 16 16 16 16 8 8 8	*78* 6600	430
1482	*147* 8 4	*147* 100	215
1485	*73* 0 0	*73* < 35	1

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Tabelle 4 (Fortsetzung)

Prot. Nr.	Komplementbindungsreaktion						Seroneutr. Test		
								Maus	Ei
1608				*31* 32 32	*138* 32 32	*225* 0 0	*225*	250	1000
1612	*21* 0 0	*29* 16 8	*37* 8 4	*58* 8 4	*72* 4 4	*177* — —	*177*	>40	>43
1624						*6* 0 0	*6*	<100	<100
1631		*18* 0 0	*26* 16 16	*45* 32 16	*58* 32 32	*276* 0 0	*276*	1350	2000
1634			*16* 8 8	*18* 8 4	*34* 4 4	*294* — —	*294*	630	6300
1647				*10* 0 0	*37* 0 0	*246* — —	*246*	0	0
1648				*1* 0 0	*20* 32 32	*280* 2 2	*280*	3400	330
1649						*?* 0 0	*?*	0	0
1656						*?* 0 0	*?*	0	0
1657						*34* 0 0	*34*	0	0
1658						*?* — —	*?*	0	0
2145						*?* 0 0	*?*	<1,5	2,1
2156					*8* 16 16	*17* 32 32	*17*	1450	63
2157				*11* 8 8	*26* 4 8	*58* 4 2	*26*	1300	210
2162					32 32	64 64		1000	320
2165						*7* 8 8	*7*	105	210
MS						*?* 2 0	*?*	<100	<100
1372			*21* 16 16	*31* 64 64	*66*[1] 8 8	*67*[2] — —	*67* liquor	416	10

[1]) KFR im Serum
[2]) KFR im Liquor

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Die Neutralisationsteste wurden auf sieben Tage alten Eiern in den Dottersack durchgeführt. Aus dem Vergleich mit den Testen auf Mäusen ging hervor, daß beide diese Objekte in etwa 95% übereinstimmend negativ oder positiv sind, obwohl die Werte von den festgestellten Indexen oft in diesen Grenzen beträchtlich schwanken. Die Autoren empfehlen die Benützung von Eiern in der Praxis, da diese viele Vorteile gegenüber den Mäusen haben, obwohl die Titers im ganzen niedriger sind, und obwohl eine geringe Zahl der positiven Fälle ganz entgehen kann.

Die Tabellen 3 und 4 zeigen außerdem die Dynamik der Antikörperbildung während der Krankheit und der Konvaleszenz.

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SEPARATUM

ACTA MICROBIOLOGICA
ACADEMIAE SCIENTIARUM HUNGARICAE

TOMUS I FASCICULI 1—3

PINTÉR, M. und BÉLÁDI, I.

DIE ZÜCHTUNG DES TSCHECHO-SLOWAKISCHEN ENCEPHALITISVIRUS IN GEWEBEKULTUREN

BUDAPEST
1954

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DIE ZÜCHTUNG DES TSCHECHOSLOWAKISCHEN ENCEPHALITISVIRUS IN GEWEBEKULTUREN

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Eingegangen am 12. August 1953

Im Laufe der in der Tschechoslowakei im Jahre 1948 aufgetretenen Encephalitisepidemie wurden von *Hloucal* und *Gallia* [1] mehrere Virusstämme isoliert. Das epidemiologische Bild war ähnlich dem der russischen Frühjahr-Sommerencephalitis, was auch durch die Isolierung des Virus aus Zecken bekräftigt wurde [2]. Im Laufe der Untersuchung der Antigenstruktur des Virus wurde eine sehr starke serologische Ähnlichkeit zwischen dem neuen Virus und das Virus der russischen Frühjahr-Sommerencephalitis sowie das Louping-ill-Virus festgestellt [3]. Auf Grund dieser Ergebnisse kann das Virus der tschechoslowakischen Encephalitis in die Gruppe der Zeckenencephalitisviren eingereiht werden. Nach der tschechoslowakischen Mitteilung wurden auch in Ungarn Untersuchungen vorgenommen, wobei man gelegentlich einer kleineren Epidemie in der Nähe von Tatabánya im Blutserum von Patienten das Auftreten einer grossen Menge von Immunstoff beobachten konnte, durch welches das tschechoslowakische Virus neutralisiert wurde [4].

Die bereits früher bekannten Typen der Zeckenencephalitisviren, das Virus der russischen Frühjahr-Sommerencephalitis bzw. der Louping-ill-Krankheit, wurden erfolgreich in embryonalen Hühnergewebekulturen gezüchtet [5, 6]. Die vorliegende Abhandlung soll über die Züchtung des neuen Zeckenencephalitisvirus und über sein Verhalten in Gewebekulturen berichten.

Methodik

Im Laufe der durchgeführten Versuche wurde der Ri-Stamm des tschechoslowakischen Encephalitisvirus verwendet, der von *Dr. Gallia* (Prag) liebenswürdigerweise zur Verfügung gestellt wurde. Das Virus wurde in Mäusepassagen überimpft und in einem 50%igen Glyzerin-Puffergemisch bei —20° C im Eisschrank gehalten.

Zur Züchtung des Virus gelangten Maitlandkulturen zur Verwendung, die auf folgende Weise hergestellt wurden. Aus dem Gemisch von drei Teilen Simms-X_6-Lösung und einem Teil Rinderserum-Ultrafiltrat wurden 4 ml in

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einen Erlenmeyerkolben von 100 ml eingemessen, wobei die Nährlösung 10 Einheiten Penicillin und 10 γ Streptomycin je ml enthielt. Aus den zerstückelten und gewaschenen Geweben von neuen-zwölftägigen Hühnerembryonen wurden ungefähr 0,2 g in einen Kolben überführt. Die Infektion der ersten Kultur erfolgte mit 0,1 ml einer 10%igen Suspension von Mäusegehirnen, die mit dem Virus infiziert waren. Die Kulturpassagen wurden alle 72 Stunden mit je 0,4 ml der Zuchtlösung durchgeführt und die Kulturen auf 37° C gehalten.

Zur Titrierung des Virus gelangten 10fache Verdünnungen zur Verwendung der in ihrer Nährlösung homogenisierten Kultur. Die Titrierung des Virus wurde mit drei Methoden ausprobiert. Zuerst wurden gleich grosse Mäusegruppen mit je 0,03 ml der Verdünnungen der Virussuspension intrazerebral beimpft. Die hierzu benutzten braunen Mäuse waren 4 bis 6 Wochen alt. Der Titer des Virus wurde in DL_{50}-Werten ausgedrückt, die nach der Formel von *Reed* und *Muench* [7] berechnet wurden. Die Wertbestimmung des Virus wurde auch durch Infizierung von bebrüteten Eiern durchgeführt. Hierbei wurden 0,2 ml der Verdünnungen der Virussuspension nach dem Verfahren von *Cox* über den Luftsack in den Dottersack der 6—8 Tage alten Eier geimpft. Die Feststellung des Titers erfolgte nach der vorigen Methode aus dem Verhältnis der abgestorbenen Embryonen. Schliesslich wurde versucht, das Virus durch Beobachtung des Wachstums der in Hängetropfen explantierten Gewebsstücke zu titrieren. Hierzu wurde das Verfahren, das *Cserey-Pechány* und Mitarbeiter [8] zur Titrierung des Virus der Aujeszkyschen Krankheit ausgearbeitet hatten, in folgender Weise angewandt. In Röhrchen von 10 mm Durchmesser wurden je 2 ml Nährlösung eingefüllt. Aus dem Virus wurde nun eine Verdünnungsserie hergestellt und danach in jedes Röhrchen 6—8 Stückchen zerkleinerter Herzen von 10—12 Tage alten Hühnerembryonen gegeben. Nach 72 Stunden wurden die Herzmuskelstückchen in Hängetropfen aus Hühnerplasma und Embryonenextrakt explantiert. Die Explantate befanden sich dann 96 Stunden hindurch bei 37° C, wobei ihr Wachstum täglich kontrolliert wurde.

Ergebnisse

Die gebrauchten Virusstämme wurden mehrere Male vor den Gewebekulturversuchen in Mäusen titriert. Die ermittelten DL_{50}-Werte schwankten in jedem Falle zwischen 10^{-6} und 10^{-8}. Der Titer der zur Infektion der ersten Gewebekultur dienenden Virussuspension betrug $10^{-6{,}23}$. Das Virus wurde über 72 Passagen gezüchtet und dabei mehrmals mit verschiedenen Methoden titriert. Nach den Ergebnissen der in Mäusen durchgeführten Messungen zeigte der Titer des gezüchteten Virus gewisse Schwankungen, wobei er jedoch kein einziges Mal einen Wert von 10^{-4} übertraf; er war also immer wesentlich niedriger als der Titer des Mäusepassagevirus. Die Resultate der in Mäusen durchgeführten Titrierungen sind aus Tabelle I ersichtlich.

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Tabelle I

Titrierung des in embryonalen Hühnerkulturen gezüchteten tschechoslowakischen Encephalitisvirus in Mäusen

Passage Nr.	Virusverdünnung					DL_{50}
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	
5.	4/4	4/4	0/4	0/4	0/4	$10^{-2.5}$
15.	4/4	3/4	0/4	0/4	0/4	$10^{-2.8}$
22.	4/4	4/4	0/4	0/4	0/4	$10^{-2.5}$
30.	4/4	2/4	0/4	0/4	0/4	$10^{-3.0}$
59.	4/4	3/4	3/4	2/4	1/4	$10^{-3.7}$
69.	4/4	4/4	2/4	1/4	0/4	$10^{-3.2}$

Zähler = Zahl der eingegangenen Mäuse
Nenner = Zahl der beimpften Mäuse

Dieselbe Erscheinung konnte bei der Züchtung des Virus aus einer neuen Kulturserie beobachtet werden. Die mit dem aus Gewebekulturen gezüchteten Virus beimpften Mäuse gingen unter den für die Infektion charakteristischen Symptomen einer schweren Nervensystemstörung ein, doch zog sich die Inkubationszeit an Stelle der normalen 4—5 Tage über 7—12 Tage hin.

Zur Feststellung, ob sich die Senkung des Virustiters infolge einer Adaptation an das Hühnergewebe nur auf Mäuse bezieht, wurde die Titrierung durch Infektion von bebrüteten Hühnereiern versucht. Die durch Impfung in den Dottersack infizierten 6—8 Tage alten Embryonen gingen infolge der Wirkung des Virus vorschriftsmässig zwischen dem 3. und dem 6. Tage nach der Infizierung ein, so dass die Titrierung des Virus auch mit dieser Methode brauchbare Resultate ergab. Der Virustiter betrug auch bei Anwendung dieser Methode einen Wert unter 10^{-4}, analog wie bei der parallel in Mäusen durchgeführten Titrierung (vgl. Tabelle II). Die infolge der Infektion eingegangenen Embryonen

Tabelle II

Titrierung des in Hühnerembryonalkulturen gezüchteten tschechoslowakischen Encephalitisvirus in Mäusen und in bebrüteten Hühnereiern

Passage Nr.	Mäuse					DL_{50}	Befruchtete Hühnereier					DL_{50}
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	
59.	4/4	3/4	2/4	2/4	1/4	$10^{-3.7}$	4/4	4/4	4/4	1/4	0/4	$10^{-3.6}$
69.	4/4	4/4	2/4	1/4	0/4	$10^{-3.2}$	4/4	4/4	2/4	0/4	0/4	$10^{-3.0}$

Zähler = Zahl der eingegangenen Mäuse bzw. Embryonen
Nenner = Zahl der beimpften Mäuse bzw. Embryonen

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zeigten die auch in Verbindung mit anderen Encephalitisviren beobachteten charakteristischen Veränderungen (Hämorrhagie, Ödem).

Die Titrierung des Virus wurde auch mit explantierten embryonalen Herzstückchen versucht. Auf die Anwesenheit des Virus wurde aus dem Auftreten oder Ausbleiben des Wachstums der Fibroblasten geschlossen. Auf Grund der in zahlreichen Fällen durchgeführten Titrierungen konnte die Feststellung gemacht werden, dass eine genaue Ermittlung des Virustiters mit dieser Methode nicht möglich ist. In einer Virusverdünnung von 1 : 10 traten in mehreren Fällen keine Fibroblasten auf, während ein andermal das Virus auf das Wachstum der Fibroblasten anscheinend keinen Einfluss ausübte. Die bei zahlreichen Viren zu beobachtende und für die Viruswirkung als charakteristisch angesehene Zerstörung und Desintegration der Fibroblasten wurde in keinem einzigen Falle wahrgenommen.

Um zu entscheiden, ob die Virusvermehrung im Herzmuskelgewebe möglich ist oder ob die Vermehrung an andere Gewebe gebunden ist, wurde die Züchtung in Kulturen vorgenommen, die entweder nur Herzmuskelstückchen oder nur Gehirngewebe enthielten. Das Virus wurde in beiderlei Kulturen über 15 Passagen gezüchtet. Der Titer sowohl des in Gehirngewebe als auch in Herzmuskeln gezüchteten Virus ergab bei Bestimmung durch Mäusetitrierung vollständig identische Werte [10^{-2}). Der Einfluss der so gezüchteten Viren auf das Wachstum der Explantate war, ähnlich wie im vorhergehenden Versuch, ganz unregelmässig.

Diskussion

Auf Grund der hier beschriebenen Versuche kann das Virus der tschechoslowakischen Encephalitis, ähnlich wie die übrigen Encephalitisviren ohne Schwierigkeiten an embryonales Hühnergewebe adaptiert und darin gut gezüchtet werden. Das gezüchtete Virus weist aber einen wesentlich niedrigeren Titer auf, als das Mäusepassagevirus. Dies ist deshalb überraschend, weil das Virus der serologisch und pathologisch sehr nahestehenden russischen Frühjahr-Sommerencephalitis auch in Gewebekulturen einen hohen Titer erreicht [9]. Eine ähnliche Titerabnahme bei der Züchtung wurde auch beim Virus der St. Louisschen Encephalitis beobachtet [10], während das Virus der equinen Encephalomyelitis auch in Gewebekulturen eine starke Vermehrung zeigt [11]. Die Titerabnahme wurde auch mit Titrierungen in bebrüteten Hühnereiern nachgewiesen. Dies bedeutet, dass man es mit einer tatsächlichen Titersenkung des Virus zu tun hat. Die pathogene Wirkung auf die Hühnerembryonen kann als identisch mit der Wirkung der anderen Encephalitisviren, in erster Linie des Louping-ill-Virus betrachtet werden [12].

Es gelang nicht, das Virus mit Herzmuskel-Gewebsexplantaten zu titrieren, trotzdem sich das Virus auch im Herzmuskel gut vermehrte. Diese Eigenschaft

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stimmt wieder mit dem Verhalten des St. Louisschen Virus überein, im Gegensatz zum Virus der equinen Encephalomyelitis, welches auch mit der obigen Methode gut titrierbar ist [11]. Über die Wertbestimmung des Virus der russischen Frühjahr-Sommerencephalitis mit Gewebsexplantaten standen keine Angaben zur Verfügung.

ZUSAMMENFASSUNG

1. Es gelang, das Virus der tschechoslowakischen Encephalitis in embryonalen Hühnergewebekulturen von Maitlandschem Typus zu züchten und über 72 Passagen zu vermehren.

2. Der Titer des gezüchteten Virus war niedriger als der Titer des Mäusepassagevirus; er schwankte zwischen 10^{-2} und 10^{-4}.

3. Es gelang nicht, das gezüchteto Virus mit explantierten Herzmuskelstückchen von Hühnerembryonen zu titrieren, dies war nur mit Mäusen und bebrüteten Eiern möglich.

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РАЗВЕДЕНИЕ ВИРУСА ЧЕХОСЛОВАЦКОГО ЭНЦЕФАЛИТА В ТКАНЕВОЙ КУЛЬТУРЕ

М. Пинтер и И. Белады

Резюме

1. Авторам удалось культивировать вирус чехословацкого энцефалита в культурэ зародыша цыпленка (тканевая культура типа Майтланд) и провести 72-кратний пассаж вируса,

2. Титр вируса культивированного таким образом был ниже титра вируса полученного пассажом у мышей и равнялся с 10^{-2} до 10^{-4},

3. Титрование культивированного вируса в культуре ткани сердечной мышцы цыпленка не удалось; положительный результат получен только в экспериментах на мышах и зародышах цыпленка.

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STUDIES ON THE OCCURRENCE OF POLIOMYELITIS VIRUSES IN HUNGARY

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STUDIES ON THE OCCURRENCE OF POLIOMYELITIS VIRUSES IN HUNGARY

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The method introduced by ENDERS et al. [1, 2, 3] for the cultivation of poliomyelitis virus has made it possible to perform a more detailed study on the occurrence of this virus in Hungary, in order to obtain more accurate data concerning its rôle in this country. The investigation consisted in isolating and typing of strains and demonstrating the presence of specific antibodies.

Methods

Our method of tissue cultivation was essentially similar to that of ROBBINS et al. [3]. Skin and muscle of embryos from the 2nd to 4th month of pregnancy were cultivated in a thin layer of plasma at 36° C. in stationary cultures. The nutrient fluid consisted of 10 per cent chicken embryo extract, 5 per cent horse serum and 85 per cent HANKS' balanced salt solution, containing streptomycin and penicillin 50 μg each per ml.

Before inoculation of the cultures the nutrient fluid was removed and replaced by amounts of 0,2 ml of the inoculum. After about 30 minutes of contact, 1,8 ml of nutrient fluid was added and the cultures were put back into the incubator. The cultures were usually inoculated after 3 to 4 days of preliminary incubation. The fluid was changed every 4 or 5 days if observation was prolonged, or if indicated by the lowering of the pH. The nutrient fluid used for the second and further changes contained only 2,5 per cent horse serum and 5 per cent embryonic extract.

Isolation of the virus was attempted in most instances from faecal and occasionally from spinal cord samples. The 10 per cent suspensions of faeces were clarified by centrifugation first at 3000 then at 8000 r. p. m. To the supernatants thus obtained penicillin and streptomycin were added to yield concentrations of 1 mg and 2 mg per ml, respectively. From the spinal cord samples a 10 per cent suspension was made, and after a single centrifugation the supernatant was used for inoculation.

Neutralisation tests were carried out with a 1 : 10 final dilution of immune serum against different dilutions of the virus. After standing at room temperature for an hour, 0,2 ml of each serum-virus mixture were inoculated into tissue cultures. Sera neutralising 100 or more tissue culture LD_{50} of virus were regarded as positive.

In virus isolation experiments the cultures were observed for 8 to 10 days. From cultures not presenting any cytopathogenic lesions, subpassages were performed with pooled supernatants harvested on the 4th and 8th days. In titration and neutralisation experiments the final results were read on the 6th day.

Immune sera for typing purposes were prepared in rodents. Type I and III poliomyelitis viruses were administered intravenously to rabbits, in the form of tissue culture fluid. Seven inoculations were made, each with 10 ml, at intervals of five days. The animals were bled on the 10th day following the last inoculation. The neutralisation indices of the immune sera thus obtained were higher than 1000. Antibodies against type II poliomyelitis viruses were produced in guinea pigs. As a vaccine, a suspension made from the brain and cord of mice infected with the Lansing strain was used, in doses of 1 ml, given intraperitoneally at three instances. The neutralisation indices of the sera obtained ranged from 100 to 1000.

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Strains of virus

In immunisation experiments and neutralisation tests the Brunhilde and Leon strains of type I and III poliomyelitis viruses were used. The specificity of the immune sera was controlled later with the Mahoney and Saukett strains. For production of type II immune sera the Lansing strain was used. Control and neutralisation tests in this case were performed with the MEF1 strain. The strains Brunhilde, Mahoney, MEF1, Leon and Saukett were kindly supplied by the Virus Department of the State Institute of Hygiene (Budapest), in the form of tissue culture supernatants. The Lansing strain was obtained from DR. GALLIA (Prague).

Results

The isolation of poliomyelitis virus was attempted from a total of 54 samples. Of these, 48 were faeces from acute patients and 6 were spinal cords obtained from fatal cases. Samples were inoculated into 4 to 6 tissue culture tubes each and two successive passages were always made. Only the results of the third passage were evaluated. Isolation was attempted at least twice from each sample.

Eighteen faecal samples out of a total of 48, were found contaminated with bacteria in spite of the addition of streptomycin and penicillin. 22 bacteriologically sterile samples exerted a more or less marked toxic effect on tissue cultures. As from these toxic materials we succeeded in isolating 7 strains of poliomyelitis virus while from the 8 non-toxic ones 2 strains, it seemed that there was no difference in the frequency of successful isolations according to the toxic or non-toxic properties of the samples. 2 of the 6 spinal cord samples were found to be contaminated with bacteria, and from the remaining 4 samples 1 strain was isolated.

Table I

Neutralisation test for typing the cytopathogenic agents isolated from patients with poliomyelitis

Strains isolated	Immune sera		
	Type I	Type II	Type III
F 0	—	—	pos.
G 15	—	pos.	—
F 30	pos.	—	—
F 32	pos.	—	—
F 36	pos.	—	—
F 38	pos.	—	—
F 39	—	pos.	—
F 46	—	pos.	—
F 49	—	pos.	—
F 50	—	pos.	—

pos. : Neutralisation test positive
— : Neutralisation test negative

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The cytopathogenic agents thus isolated were serially transferred and from time to time titrated. In spite of the passages, the titre of some strains never exceeded 10^{-2}, while that of the others reached 10^{-5}. Isolated strains were immediately inoculated intracerebrally into white mice, but no symptoms were observed. Suckling mice proved equally insusceptible to these viruses.

It has been attempted to identify the strains by means of the immune sera available. The results of repeated neutralisation tests, summarised in Table I, clearly show that all the strains isolated were poliomyelitis viruses. Out of the 10 strains isolated 4 belonged to type I, 5 to type II, and 1 to type III.

Parallel with the virus isolation experiments, studies concerning the frequency of antibodies against the different types of poliomyelitis viruses in healthy persons were also carried out. The results of the neutralisation tests carried out with the three types of poliomyelitis viruses and the sera of persons of different age groups are summarised in Table II.

Table II

The frequency of occurrence of neutralising antibodies against the three types of poliomyelitis virus in healthy persons

Age group	Number of subjets	Type I		Type II		Type III	
		positive	per cent	positive	per cent	positive	per cent
$^1/_2$—2 years	21	6	28	2	9	4	19
3—7 years	29	17	59	13	45	16	55
8—15 years	39	23	59	29	74	21	54
16—30 years	37	27	73	30	81	25	67

It can be seen from Table II that the frequency of occurrence of antibodies increases with age. A parallelism in the occurrence of type I and III antibodies could be observed while that of type II exhibited a somewhat different pattern.

Discussion

The investigation was carried out in order to attempt isolation of poliomyelitis virus in human embryonic tissue cultures. Ten strains could be isolated, namely 9 from faeces and 1 from a spinal cord. Only from one third of the adequate faecal samples was the isolation successful. This ratio is especially low, as most of the samples have been obtained from typical cases of poliomyelitis. As to the cause of the low percentage, transport of the samples, often exceeding the time desirable, might have caused a lowering of the virus content. However, the sensitivity of the method employed is not the highest

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either, as, according to some more recent data, the probability of isolation is higher in monolayer epithelial than in the usual fibroblast cultures [4].

Before the present studies, only the isolation of type I poliomyelitis virus had been reported in Hungary [5]. It was remarkable that, out of the 10 strains isolated during the epidemic in 1954, 5 belonged to type II. Since 4 strains of type II were isolated from patients admitted at the same time to the Department of Paediatrics at Szeged, there was probably a local accumulation of type II infections. A similar observation has been made by ROBBINS et al. [6] who, out of 13 strains isolated, identified 10 strains as belonging to type III.

From the results of the neutralisation tests, it could be established that the conditions of infection in Hungarian towns are similar to those prevailing in other civilized countries. Our studies carried out with the sera of healthy inhabitants of Szeged have shown that the appearance of antibodies against type I and III poliomyelitis viruses exhibits an intimate parallelism. Characteristically the occurrence of antibody does not change in the age groups between 3 and 15 years, since it attains the 50 per cent value before the third year. It seems, therefore, that most of the infections with these two types are contracted in early childhood. The frequency of type II antibodies in the sera tested was more regularly related to the age than that of the other two types, and was in good agreement with our earlier observations made in 1951 by Lansing virus neutralisation tests in mice [7].

Summary

1. We have succeeded in isolating in 1954 10 poliomyelitis virus strains in Hungary from patients with poliomyelitis, by making use of human embryonic tissue cultures. Nine of the strains have been isolated from faeces and one from a spinal cord. Typing was made by immune sera, produced in the guinea pig for type II and in rabbits for types I and III. Four strains were found to belong to type I, five to type II and one to type III.

2. Neutralising antibodies against all the three types have been demonstrated at a varying ratio in the sera of healthy persons. The frequency of occurrence of the antibodies rose with age. In the adult population ; 73 per cent were type I, 81 per cent type II, and 67 per cent were type III strains.

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ИЗУЧЕНИЕ ВСТРЕЧАЕМОСТИ ВИРУСОВ ПОЛИОМИЭЛИТА В ВЕНГРИИ

М. ПИНТЕР, Э. АБРАХАМ и М. РАВНАИ

Резюме

1. От больных полиомиэлитом в Венгрии в 1954 году удалось изолировать десять штаммов вируса полиомиэлита в культурах из человеческой эмбриональной ткани. Материалом исследования в девяти случаях служили испражнения, а в одном случае — спинной мозг. Штаммы могли быть типизированы в тканевых культурах с помощью иммунных сывороток, полученных от морских свинок (тип 2.) и от кроликов (тип 1. и 3.). К типу 1 относятся четыре штамма, к типу 2 – пять штаммов и к типу 3 – один штамм.

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