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Consideration on the Mechanism of Occurrence of Tubercle Bacilli Positive on Direct Examination but Negative on Culutre

Yoshio TAKAHASHI

A bacteriological study has been done on 331 pulmonary fragments resected from patients with pulmonary tuberculosis. The results have led to the following consideration.

1. Tubercle bacilli in pulmonary lesions undergo a certain definite morphological and physiological alteration as the environment becomes unfavorable for their existence. They thus transform from the normal bacillary form into the clumpy, beaded and granular forms. The acid-fastness is gradually lost with the transformation.

2. It is very likely that transformed tubercle bacilli in lesions return to the normal bacillary form, when the environment becomes favorable for their multiplication. Therefore, the transformation of tubercle bacilli in lesions is not merely a phenomenon of destruction: it is rather a phenomenon of resistance. The author considers the above mentioned transformed form of tubercle bacilli in lesions, especially of granular form, as resistant forms which the bacilli take to resist unfavorable environment.

3. Tubercle bacilli positive on direct examination but negative on culture are detectable more frequently in stationary lesions than in progressive ones. Morphologically, they are frequent in the transformed forms, especially in the beaded and granular forms. Tinctorially, they are so in the cyanophilic form.

4. Contrary to these facts just mentioned, the frequency of drug-resistance of tubercle bacilli in lesions is great in the normal bacillary acid-fast form and small in the transformed forms. Drug-resistance is hardly noticed in the granular form.

5. From all the facts above mentioned, it seems very likely that the mechanism of occurrence of smear-positive, culture-negative tubercle bacilli in pulmonary lesions is identical to the so-called L-form of other bacterial species.

Studies on the Antigen-Antibody Reaction in Agar

I. Precipitin reaction of tuberculous sera by Oudin's agar diffusion technique.

Masuwo ITAKURA, Tadashi IMAI and Kazuwo TAKAHASHI

Oudin's agar diffusion technique for precipitin reaction was carried out for tuberculin with special reference to the mode of appearance of precipitation bands and the antigenic complexity of tuberculin was confirmed.

1) Antigenically tuberculin consist of at least ten components. The number of the bands formed in agar depends on the concentrations of antigen and antisera tested or on the length of time of diffusion.

In this experiment, precipitation bands were observed many more in number when a ten fold dilution of tuberculin was used as antigen than when an undiluted tuberculin was used.

2) The distance from the interface to which the advancing edge of a band travels is proportional to the square root of the time and the logarithm of the antigen concentration. This relation, however,

deviates to some extent when too concentrated or too diluted antigen is used or when the time of diffusion prolonged.

Biological Study of *Mycobacterium Tuberculosis*
with Special Reference to Biological Differences between Virulent
and Avirulent Tubercle Bacilli.

I. Relation of the age by days of the bacilli to their viability,
dehydrogenase activity, and respiration.

Toshio YOKOI

Strains of virulent human tubercle bacilli, Nakano and Aoyama B, a strain of attenuated human tubercle bacilli, Imamura, a BCG strain, Yoken, a strain of avian tubercle bacilli, Takeo, and *M. phlei* and *M. smegmatis* were examined at intervals for their viability, oxygen consumption, and dehydrogenase activity during incubation. The results obtained were as follows.

1) Generation time of the bacilli.

Generation time of *M. phlei*, *M. smegmatis*, and the avian bacillus was about 3.2 to 3.5 hours, while that of strain Imamura, Aoyama B, Nakano and BCG was 19.3, 20.8, 26.5 and 16.8 hours respectively.

2) Viability of the bacilli.

The attenuated tubercle bacilli, Imamura and BCG, of which the *in vivo* viability was weak, revealed also a weak viability *in vitro*. The viability of the virulent bacilli, strains Nakano and Aoyama B, lasted for a relatively long period of incubation, while that of strain Imamura and BCG decreased rapidly at the early stage of culture. The viability of *M. phlei* and the avian bacillus was almost similar to that of strains Nakano and Aoyama B.

3) Dehydrogenase activity of the bacilli.

In the absence of any substrate, no difference was observed in dehydrogenase activity among all the strains except *M. phlei* which maintained a markedly high activity. In the presence of lactate, remarkable differences were observed between virulent strains, Nakano and Aoyama B, on the one hand, and attenuated strains Imamura and BCG on the other ; the activity of the former strains lasted without marked decrease till the late stage of incubation, while that of the latter rapidly decreased at the early stage of incubation.

As for the enzymatic activity of young cultures of all the bacilli used, strains Nakano and Aoyama B and *M. phlei* were found to be the highest and that of BCG the lowest.

4) Endogenous respiration and respiration in the presence of lactate.

Among the young cultures of the bacilli used, endogenous respiration and respiration in the presence of lactate of *M. phlei*, *M. smegmatis* and *M. avium* were much intenser than that of strains Nakano, Aoyama B, Imamura and BCG which all showed similarly a low oxygen consumption.

Oxygen consumption of strains Imamura and BCG decreased more rapidly to an unmeasurable degree than that of strains Nakano and Aoyama B.

Oxygen consumption of the *avium* bacillus and *M. phlei* remained comparatively high till the late stage of incubation.

Studies on the Metabolism of *Mycobacterium Tuberculosis*
I. Effects on the growth of BCG of carbohydrates, compounds
related to the Krebscycle and fatty acids.

Tadashi IMAI

By the use of the small inoculum technique, the generation time was determined for BCG grown in Kirchner's basal medium in which glycerol, the only carbon source, had been replaced by carbohydrates, compounds relating to TCA cycle or fatty acids. This was done in order to investigate whether or not such compounds can be utilized by BCG as the carbon source. The harvest of BCG were measured. The results were as follows:

i) Of the carbohydrates and polyhydric alcohols used, only glycerol, glucose and maltose were utilized for the growth of BCG.

ii) Of the compounds related to the Krebs tricarboxylic acid cycle, pyruvic, α -ketoglutaric, lactic and acetic acid supported the growth of BCG, while cis-aconitic, citric, succinic, fumaric and l-malic acid did not.

iii) Of the fatty acids, acetic, propionic, lactic, caproic, n-caprylic and lauric acid supported the growth of BCG in varying degrees, while formic, palmitic and oleic acid did not. These fatty acids supported the growth only in a narrow range of their concentrations. Caprylic acid (10 carbon atoms) was most favorable for the growth. Still higher fatty acids were hardly favorable and lower ones prolonged the generation time, thus resulting in a lesser harvest.

Correlation between Virulence of Tubercle Bacilli
and Stimulation of Oxygen Uptake by Lactic Acid and by
Short-Chain Fatty Acids

Koji SATO

The influence of L (+) lactic acid upon the oxygen consumption of resting cells of various *Mycobacteria* was examined at various levels of pH, on different days of cultivation and under different cultural conditions. The first four members of the short-chain fatty acid series were also tested for their stimulative activity upon the oxygen consumption of various *Mycobacteria*. The data obtained are as follows:

1) Stimulation of oxygen consumption by lactic acid was greatest at any pH for the saprophyte *M. phlei* and was least for the pathogen H37Rv.

M. smegmatis, A71 strain of *M. avium*, BCG, Imamura strain and Nakano strain of human tubercle bacilli were found in this order to range between *M. phlei* and H37Rv. The optimal pH for *M. phlei* and *M. smegmatis* to oxidize lactic acid stands below 7.0.

2) Stimulation of oxygen consumption by lower fatty acids was greatest for *M. smegmatis* and *M. phlei*, moderate for A 71 strain of *M. avium* and BCG, and least for H37Ra and H37Rv of human tubercle bacilli. Of the last two strains, oxygen consumption of the latter strain was less stimulated by the lower fatty acids than was the case with the former strain.

No definite correlation was found between the number of C atoms of the fatty acids tested and tested and their stimulation of oxygen uptake upon *Mycobacterisa*.

3) Stimulation by lactic acid of the oxygen consumption of H37Ra strain of human tubercle bacilli is always greater than that of H37Rv, regardless of their age or cultural conditions.

4) It may be possible, to some extent, to get information of the degrees of virulence of various strains of tubercle bacilli by comparing the vigor of their oxidative attack upon lactate.

Influence of Egg-Yolk on the Immunogenicity of Heat-Killed Tubercle Bacilli

Ken-ichi YAMAMOTO, Katumi AWA and Shigetoshi TAKAGI

It was shown in a previous paper that, when guinea pigs were vaccinated with BCG suspended in egg-yolk solution, tuberculin allergy and acquired resistance to tuberculosis were intensified. This was attributed to the possible in vivo protective action of egg-yolk on BCG.

The present paper deals with the influence of egg-yolk on the tuberculin allergy and acquired resistance to tuberculosis induced in guinea pigs by vaccination with heat-killed tubercle bacilli incorporated in egg-yolk solution,

Guinea pigs were divided into two groups of equal number, one receiving subcutaneously 10 mg of heat-killed virulent tubercle bacilli, strain Nakano suspended in distilled water and the other tuberculin the same dose of heat-killed bacilli suspended in 50 per cent egg-yolk solution. After vaccination, the skin reaction was examined and recorded weekly. Seven weeks after vaccination all the animals, together with the control animals, were challenged subcutaneously by the injection with 0.01 mg of virulent tubercle bacilli, strain Nakano. They were sacrificed nine weeks after challenge and a comparative observation was made grossly as well as histologically of tuberculous lesions in the visceral organs and the lymph nodes. Quantitative cultures were made for comparison of the bacilli in spleens of all the animals.

The experiment led to the following results.

1. No appreciable difference in tuberculin allergy was observed between these two vaccinated animal groups.

2. Acquired resistance to tuberculosis was found to be slightly stronger in case of vaccination with the bacilli suspended in egg-yolk solution than that with heat-killed tubercle bacilli suspended in distilled water.

Daily Fluctuation in the Drug-Resistance of Tubercle Bacilli in Sputa

Yoshitame NAGAYAMA, Koji MOCHIZUKI, Kotaro TAKEDA,
Kazuhiko DOI, Seiichi FUJITA and Tamotsu YOSHIDA

By means of the direct method for evaluating drug-resistance of tubercle bacilli, using 1 percent Ogawa's media, drug-resistance was examined consecutively for 10 days for tubercle bacilli present in sputa from 20 patients with pulmonary tuberculosis who all had more than 2 cavities and in whose sputa tubercle bacilli had been recorded to have a complete resistance of 10^7 /ml against one or more of three chemotherapeutica: streptomycin, isoniazid and PAS. The study led to the following results:

1. In only a small number of the cases, drug-resistance revealed itself as always identical in the course of the observation of 10 days; in the majority of the cases, considerable daily difference were noticed in drug-resistance. Cases in which drug-resistance did not coincide with 10^7 /ml were 10 for streptomycin (50%) and 17 for PAS. (85%)

2. Tendency was noticed that drug-resistance reveals itself rather as incomplete, when only a small number of colonies have grown on control tubes of medium, when a sufficient number of colonies have grown on control tubes, drug-resistance reveals itself almost as complete.

3. When cases showing for more than one day a complete resistance against 10^7 /ml of the drugs were taken as complete resistant cases were to be recorded as such by means of the examination of only one day, it was 52% for streptomycin and 38% for PAS.

4. The data above mentioned indicates clearly that examining sputa for at least consecutive 3 days is needed the exact drug-resistance of tubercle bacilli in these materials can be recorded.

Pathogenic Effect of "Cord Factor" on Mice
—A Histo-Pathological Study

Yoshito SHIMMYO, Isao SEKIKAWA, Shigetoshi TAKAGI,
Toyaji KOBAYASI & Tetsuji HASHIMOTO

The pathogenic effect of the "cord factor" (Bloch) on mice was histologically studied.

The experiments and the results were as follows:

1. A first group of mice were intraperitoneally injected with 2 mg of cord factor 8 times at two day's interval. The mice showed severe hemorrhage, hyperemia and slight alveolitis in the lungs. Acute splenitis and karyorrhexia of the lymphocytes were observed in the spleen. The peritoneal wall showed intensive necrotic inflammation with a severe exudation of polymorphonuclears. The liver showed dilatation in the sinusoid in which leukocyte thrombus was found.
2. A second group of mice previously inoculated with BCG were injected with the factor in the same manner. The mice showed lesions similar to those in the first group. However, at the end of the experiment the intensity of the lesion was slighter than in the first group.
3. As control, a third group of mice were intraperitoneally injected with paraffine oil, solvent of the cord factor. The mice showed only slight lesion in the peritoneal wall.
4. A fourth group of mice were injected with the factor just in the same manner as in the first group, but subcutaneously. A fifth group of mice were intraperitoneally injected with a single dose of 20 mg of cord factor. Qualitatively these animals showed lesion similar to that in those of the first group, but quantitatively it was much less severe.

Influence of Antiplasmic Drug on Tuberculosis

1. On tuberculosis experimentally produced in rabbits

Tetsuji HASHIMOTO, Mikio ITO, Takashi SASAKI & Hiroshi TOKITA

Rabbits previously immunized with BCG and normal rabbits were injected intravenously with virulent human tubercle bacilli. From 3 days before injection a half of these animals received subcutaneously an antiplasmic drug (Ipsilon, DAIICHI SEIYAKU Co.) daily until the end of the experiments.

The animals were sacrificed 10, 20 and 40 days after injection, and the visceral organs were studied histologically and bacteriologically. The experiments led to the following results:

An inhibition, though slight but specific, of the intensity of tuberculin reaction, occurred by injection with the drug. The numbers of viable bacilli in the spleen were somewhat smaller in the animals injected with the drug than in non-treated animals. The effect of the drug on tuberculous lesion was qualitatively almost equal in the immunized and non-immunized animals. The lesions, especially exudative ones, in the animals injected with the drug was weaker than in control animals. However, at the end of the experiments, this difference could be found no more.

From these results the antiplasmic drug was found to accelerate the course of the tuberculosis experimentally produced in rabbits.

Influence of the Wax Fraction of Tubercle Bacilli
on the Arthus Phenomenon

Takashi SASAKI, Mikio ITO, Shigetoshi TAKAGI & Hidetoshi KOSUGI

In order to study the influence of the wax fraction of tubercle bacilli on the Arthus phenomenon, one group of rabbits were injected with the bacillary wax mixed with ovalbumin. As control, a second

group of rabbits were injected with ovalbumin and the third with the wax. Ten days and thirty days after the last injection of sensitization all the rabbits were reinjected intracutaneously with ovalbumin solution as well as with old tuberculin. The skin reactions thus produced were measured at short intervals during 72 hours after the reinjection. In every case, immediately before injection sera were obtained from the animals and studied electrophoretically. The experiments led to the following results:

- 1) In the animals of the first group, which had received the mixture of wax and ovalbumin, the Arthus phenomenon was most intensive.
- 2) In the animals of the third group, which had received the bacillary wax only, no positive skin reaction occurred.
- 3) The skin reaction attained its peak 24 hours after reinjection, except in the animals of the second group on the 10th day after sensitization, in which the peak was at the 10th hour.
- 4) In all the animals no positive skin reaction was observed following injection of old tuberculin.
- 5) The quantity of the serum γ -globulin was strikingly increased in the animals of the first and second group, especially in those of the first group.
- 6) Histologically, at the earlier stage of the reaction polymorphonuclear leukocytes were always predominant in the skin lesion, while after 48 hours mononuclears became predominant.

Pathological Study on Delayed Shock

2. Comparison of Delayed Shock with Anaphylactic Shock in View of Their Histology

Hiroshi TOKITA

Rabbits Previously sensitized with crystalline ovalbumin or sheep red cells were reinjected with the same antigen. Some animals showed severe anaphylactic symptoms and died about 10 minutes after injection. Survived animals were sacrificed 1, 2 and 24 hours after injection. The pathological study of the visceral organs led to the following results:

1. The intensity of the shock symptoms was not always in parallel with the intensity of the Arthus phenomenon as measured 2 days before injection.
2. Macroscopically, the animals which underwent anaphylactic shock showed severe pulmonary emphysema and intense hyperemia in the lungs and spleen.
3. Histologically, in the first stage of anaphylactic shock, lesions in the lungs were found mainly restricted to a narrow range around the small blood vessels: constriction of the arterioles, embolism of the vessels with hyaline substance, hemorrhage and perivascular cellular exudation. After this stage the exudative lesions became gradually severer and wider: intensive exudation of polymorphonuclears into the alveole and its walls. The blood vessels showed a marked embolism with clumped leukocytes.
4. The spleen showed severe congestion, dilatation in the sinus, in which were found a great number of polymorphonuclears and mononuclears. Twenty-four hours after injection the cells in the white pulp underwent cytolysis showing severe karyorrhexia.

The liver showed marked dilatation in the sinusoid, in which clumped leukocytes and some mononuclears were found. Twenty-four hours after injection the hepatic cells underwent degeneration and focal necrosis with an exudation of polymorphonuclears. The lymph nodes became coarse in structure owing to edema, lympholysis and, possibly, increased flowing of the lymphocytes into circulation.

5. Almost no difference was observed between the lesions of the animals injected with ovalbumin and those with sheep red cells.
6. These findings were compared with those observed in delayed shock described in the preceding paper.