Soviet Academy of Medicine (Laboratory for Infectious Pathology of the Pathological Department), Institute of Experimental Medicine, and Laboratory for Intestinal Infections of the "Pasteur" Research Institute, Leningrad

EXPERIMENTAL PNEUMONIA CAUSED BY BACTERIA OF THE SHIGELLA GROUP

M. V. Voino-Yasenetsky and M. K. Voino-Yasenetskaya

(Received January 25, 1961)

In experimental pathology two fundamental methods are employed for the study of infectious processes. Certain human infectious diseases can be reproduced fairly accurately in specially selected animals. A true experimental model of a particular disease can be said to have been produced when infection has been induced in a manner closely similar to its natural occurrence, if the experimental disease runs approximately the same course as in humans, and if the clinical and pathological manifestations closely resemble those observed in human patients. Such a model, however, can not be reproduced for all infectious diseases. Even those experimental diseases in animals which show the closest resemblance to human pathology reveal certain — sometimes essential — features that distinguish them from their prototype human disease.

The other method is to produce a specific infectious process deliberately creating conditions different from the natural occurrence of disease. This applies to intravenous, intraperitoneal, intracerebral and other modes of inoculation, as well as to the use of huge inocula, or preliminary treatment of test animals, exposed to influences, improbable or impossible under natural conditions, etc. The artificially induced infection may differ considerably from the natural disease with respect to location of lesions and clinical course. Nevertheless important data on pathogenesis, treatment and prevention have been obtained this way. In laboratory practice this method (or methods) is used extensively; it makes experimentation simpler and conclusions more obvious.

In our attempts to induce shigellosis with intestinal lesions characteristic of dysentery in laboratory animals, we were — no more successful than most investigators before us. Ingested pathogens of dysentery failed to invade the gastro-intestinal tract. Even if we did succeed now and then in provoking intestinal infection, it proved extremely difficult to observe the process in a systematic manner. However, in some experiments, made in collaboration with L. S. Dr. Bibinova, mice and rabbits were found to have developed pneumonia if the pathogens had gained access to their respiratory passages. Shigellae were isolated in pure culture from the lungs of these animals. It was this chance observation that prompted us to study the effects of intrapulmonary

inoculation of Shigellae. We were encouraged in doing so by the reports of SERÉNY in 1955 on "keratoconjunctivitis shigellosa". Intraconjunctival Shigella infection, productive of local inflammations in guinea pigs, was subsequently used by several workers (HECKEL *et al.*, 1957; MANOLOV, 1957; SIROKO, 1958; etc). This method however is not suitable for quantitative determination of the reproduction or destruction of bacteria. Besides, guinea pigs are not readily available and too expensive for mass experimentation

It was shown in our first paper on the subject (1957) that by quantitative bacteriological determinations the fate of Shigellae in pulmonary tissue of mice could be followed after intranasal inoculation, and that recently isolated Kruse—Sonne *(Sh. sonnei*, 2. FLEXNER *(Sh. flexneri 1b, 2a, 3)*, Newcastle *(Sh. flexneri)* and different types of Boyd—Novgorodskaya *(Sh. boydii)* strains were well able to survive and multiply in the lung of white mice

When Shigella cultures were maintained under usual laboratory conditions for a long time, their capacity to grow in vivo, tended to decline; althoug their other biochemical and serological properties, and even their toxicity proved to be unchanged. In contrast to the above-mentioned types, *Sh. dysenteriae 2*, smooth Kruse—Sonne strains and certain Newcastle strains fail to proliferate pulmonary tissue of animals even when inoculated directly following isolation from human patients. Similar observations were made in connection with conjunctival infection of guinea pigs (SANTARENKO and TESLA, 1960).

Intrapulmonary Shigella infection of mice has proved to be a useful tool for the study of antibiotics (VONIO-YASENETSKAYA, 1958) and may evidently serve for other purposes as well. However, in order to appreciate the scope of this experimental method, it is necessary to study every detail of the pathological changes, induced by Shigella infection in the affected animals. The purpose of this investigation was to study.

(1) Those changes (reactive processes in particular) which occur in mice at different periods following infection with various Shigella strains;

(2) Intrapulmonary loci, where Shigella bacilli proliferate, the medium, supporting their growth;

(3) The possible reason why certain strains of dysentery bacilli fail to survive in the lung of animals.

Material and method

Our experiments consisted of three principal groups, each with 20 separate series, involving more than 1100 white mice of 12 to 16 g body weight. Experiments of the first and second group were run with strains capable of proliferating in the lung of mice *(Sh. sonnei, Sh. flexneri 2a and 6, Sh. boydii 1)* and with strains which do not survive there *(Sh. dysenteriae 2.)* Eighteen hour broth cultures, containing several millions of organisms per 0.05 ml, served as inocula, introduced intranasally under light ether anaesthesia.

Several millions or a thousand millions, respectively, of *Sh. dysenteriae* 2, *Sh. flexneri* and *Sh. sonnei*, killed by heating during an hour, were used for intranasal inoculation in the third group of experiments.

Bacteriological examination (as described in our paper of 1957) were performed in the first two groups immediately after inoculation and then 24 and 48 hours later. At least 5 mice per series at each interval were used for this purpose. Isolation of bacteria was performed at 0, 3, 6, 9, 12 and 18 hours in supplementary experiments. A total of 334 mice were used for quantitative bacteriological examinations.

Histological sections were prepared from 453 mice and contact preparations from 146 further animals. The mice were killed after 15 minutes and 1, 2, 3, 6, 24 and 48 hours respectively by means of air embolism; in most series examinations were also performed at later periods (up to 20 days). Sections were stained with haematoxylin-eosin, carbolthionine and Goldman's Sudan alphanaphthol. Smears were first fixed in methylalcohol and stained after Romanowsky—Giemsa.

1. *Intranasal inoculation of mice with living Shigella strains*

Mice infected with *Sh. dysenteriae* appeared drowsy for the first hours after anaesthesia but behaved normally thereafter. Only 4 out of a total of 100 animals died within the next 3 days.

Infection with other strains of Shigella had a more serious effect. In a series of 113 animals infected with *Sh. sonnei*, the first death occurred after 7 hours, and mortality rose to 83.3 per cent by the end of the first day. In a series of 178 mice infected with *Sh. flexneri*, 17 per cent died on the first day, and 68.7 per cent of the rest on the second. No more deaths occurred thereafter.

Bacteriological results are assembled in Table 1. They display differences between various Shigella strains as regards growth in pulmonary tissue of animals.

Table 1

Number (in millions) of Shigella bacilli in mouse lungs at different times following infection
(Mean values in brackets)

Strain	Isolated after 10 to 15 minutes	After 24 hours		After 48 hours	
		Animals killed	Animals perished	Animals killed	Animals perished
Sh. dys. 2	1.2—12.4 (7.2)	0.03—1.0 (0.3)	(0.04)	0,004—0,1 (0.04)	0,04—0.07 (0.06)
Sh. sonnei	2.0—6.8 (4.6)	123.0—516.0 (237.1)	82.0—9460.0 (2398.6)		
Sh. boydii 1	1.6—11.5 (5.5)	21.0—387.5 (185.8)	200.0—300.0 (263.6)	4.8	8.0—400.0 (58.2)
Sh. flexneri 2a ..	2.6—6.9 (4.6)	0.3—222.0 (55.5)	35.6—298.0 (105.9)	0.07—2.0 (1.0)	1.4—253.0 (33.7)
Sh. flexneri 6 ...	2.0—4.0 (3.2)	2.0—65.0 (14.3)	29.6	0.007—0.4 (0.004)	0.2—44.9 (1.3)

Isolations from the lung of mice infected with highly pathogenic Kruse—Sonne bacteria and sacrificed within 24 hours, always gave approximately uniform results, while rates of proliferation of other strains known to be viable in pulmonary tissue, seemed to vary from animal to animal. The greatest fluctuations in this respect were observed in the case of Sh. flexneri. Microscopy demonstrated these features with great emphasis.

Shigella bacilli are clearly visible in the azure-eosin or thionine-stained preparations. Most of them had invaded the pulmonary alveoli and only some organisms remained on the mucosal surface of the trachea and bronchi. They had a length of 2.0 to 2.5 μ; lengths of 4 to 5 μ were rare, and bacteria of such size were generally constricted in the middle. (Shigella bacilli the contact preparations were nearly double that size, their structural details being more apparent; these are outside the scope of this presentation.)

The inoculated organisms were not evenly distributed within the lung. Most of them seemed to have settled in the alveolar ducts. Bacterial counts were higher in the apical cranial

parts of lungs: 73 to 98 organisms per 100 alveoli against 22 to 42 in the basal lobes. Organisms were scattered singly over alveolar walls, while some of them formed small groups of 3 to 10 bacilli. These accumulations showed no regular pattern.

Although the initial in the lungs of mice were uniform in various series, this uniformity soon ceased to be apparent. Observations are therefore referred to each of the main groups.

2. *Inoculation with Sh dysenteriae* 2

One of the earliest signs of the general response to intrapulmonary Shigella infection was found to be secretion by the respiratory epithelium. This was not always sufficiently vigorous to remove all bacilli from the trachea and the bronchial tree.

Alveoli are known to contain cells, capable of assuming phagocytosis which have an important protective function. It was observed by G. I. ILYIN in our laboratory that more than 90 per cent of staphylococci introduced intranasally to mice were engulfed by the alveolar phagocytes within a few hours. These phagocytes proved less active against *Sh. dysenteriae*, no more than 10 to 30 per cent of the bacteria being ingested during the first hours.

On fixation of pulmonary tissue by a special method (boiling), the appearance of a faintly staining fluid can be observed around arteries and in some of the alveoli within 15 to 60 minutes following inoculation. This fluid does not represent broth introduced with the inoculum as it is also seen if organisms were suspended in saline.

This serous fluid (which evidently contains the non-specific immune substances of the blood plasma) produces no significant effect on Shigella. The number of organisms in the alveoli may even rise after the lapse of 2 to 3 hours (Fig. 1), mitoses becoming more frequent by that time.

Accumulation of polymorph nuclear leucocytes first around the bronchi and then in the alveolar capillaries, begins at the same time. The cellular response is slight during the first hour following inoculation to become pronounced within two hours (Fig. 2). The leucocytes occupy the lumen of small blood vessels at first; later on by the 3 hour they escape from the blood-stream and seem to coat the inner surface of the alveoli. Since the volume of exudate is usually small even at a later stage of the extending inflammatory reaction, alveolar leucocytes are seen mostly about the walls. Hereupon, some of the alveoli collapse, while others become completely filled with granular leucocytes (Fig. 3), find access to the bronchi as well, though but in small numbers on the first day.

As soon as they appear in the alveoli, polymorphonuclear leucocytes begin to ingest the Shigellae. A few leucocytes, containing Shigellae in their cytoplasm, could be seen in lung sections or contact preparations as early as 2 to 3 hours after inoculation, and nearly all of the pathogens were found to have been ingested by leucocytes (and partly by macrophages) by the 6th or 9th hour. Although each polymorphonuclear is capable of phagocytizing 30 or more bacilli, they usually contained but a few bacteria. At first, phagocyted Shigellae appear as short (sometimes long) rods staining bright by the 9th hour some of them retain their original shape but they take the dye weakly so that it is quite difficult to distinguish them. Scattered debris of Shigellae were frequently observed in the cytoplasm of the leucocytes, their nucleus became considerably deformed with digestion of the invaders.

While vigorous phagocytosis proceeded successfully some of the invaders escaped (Fig. 4), remaining free to from microscopic colonies composed of a few dozen short coccus-like rods. By the end of the first day, however, bacterial counts had decreased significantly, so that lung sections and contact preparations usually contained just a few microbial fragments after another 24 hours. Most of the occasional Shigellae persisting in the bronchi, were eliminated with the products of the epithelial secretion. If any escaped, they never failed to be ingested by leucocytes.

In a single case only, in a mouse, killed 24 hours after inoculation, were Shigella bacilli found inside a few epithelial cells in the bronchi filled with polymorphonuclears. In spite of their containing 1 to 5 well-staining dysentery bacteria in the cytoplasm, these epithelial cells showed no signs of damage.

Pathologic changes, accompanying the destruction of *Sh. dys.* 2 bacteria were evidently due to release of endotoxin. Perivascular oedema and haemorrhages about certain branches of the pulmonary artery appeared as early as the 2nd hour following inoculation. Haemorrhages involving a few alveoli were only found in some mice. Spastic contraction of small arteries accompanying bronchioles was observed frequently though not regularly.

When considered together, all these pathologic phenomena, described as happening in the lung of *Sh. dys.* 2-infected mice, are characteristic of pneumonia. This pneumonia, largely focal, is most conspicuous in the upper and middle portions of the lung, *i. e.* in those areas which are selectively colonized by organisms introduced intranasally. The disease reaches its

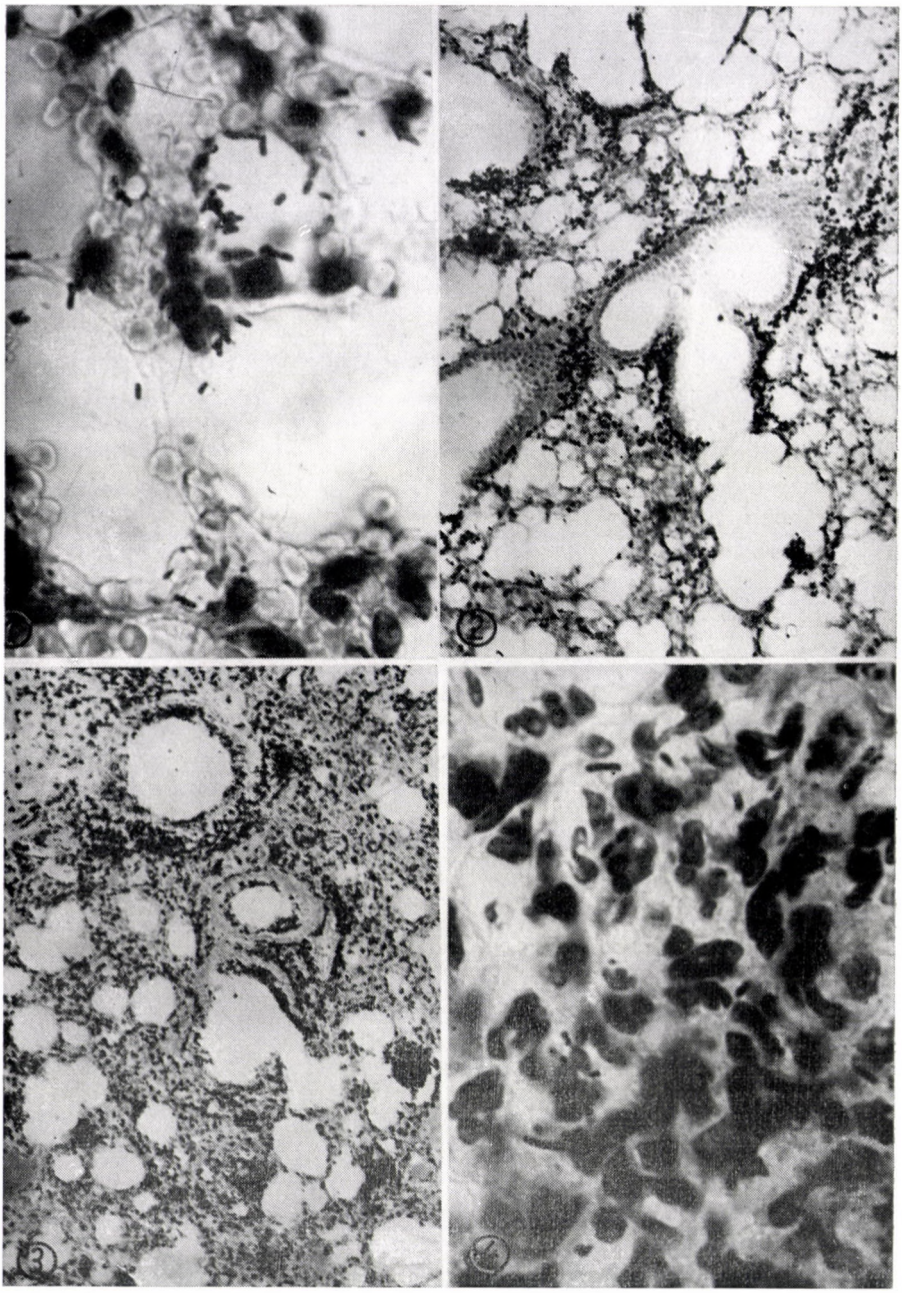

Fig. 1. Free *Sh. dysenteriae 2* in the alveoli of a mouse killed 3 hrs after inoculation. Thionine. ×120

Fig. 2. Accumulation of polymorphonuclear leucocytes (staining dark) around the bronchi, arteries and alveolar areas, 2 hrs after infection. Oxidase reaction. Goldman's-Sudan alpha-naphthol. ×100

Fig. 3. Accumulation of leucocytes in the alveoli and bronchi, 9 hrs after inoculation with *Sh. dysenteriae 2*. Goldman's dye. ×100

Fig. 4. Free and phagocyted *Sh. dysenteriae 2* in leucocytic exudate filling alveoli. Thionine, ×1350

climax by the end of the first or second day, when it is often accompanied by rather widespread atelectasis. Elimination of the exudate through the bronchi, the rest being disposed of by macrophages, has been shown to commence at the same time.

The pulmonary tissue of most animals killed on the 5th to 7th day of the experiment presented a practically normal picture. Only small groups of macrophages about alveolar ducts and accumulation of round cells about some arteries and veines betrayed the pathologic processes that had occurred here. In the atelectatic areas alveoli were observed to distend, gradually becoming airfilled.

It is only natural that the body, as a whole never fails to participate in the processes occurring in the lungs of infected mice. This is manifested morphologically as moderate general leucocytosis, revealed by leucocyte counts in the capillaries of the liver, kidneys and myocardium. The number of polymorphonuclear leucocytes in the myocardium and the renal glomeruli rises to one-and-a half or twice its original value by the third hour after infection, and either remains at this level or returns to normal. Leucocytosis is more and steadily pronounced in the

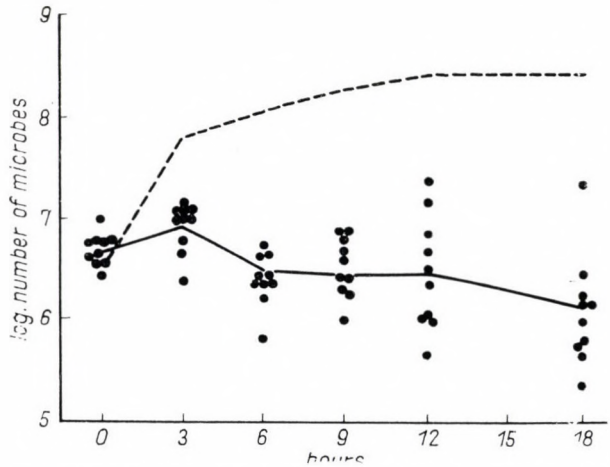

Fig. 5. Sh. dys. 2 bacteria contained in mouse lungs at different times after infection. Circles indicate number of microbes in the lung. Continuous line indicates logarithmic mean value at a given time of the experiment. Dotted line indicates the rate of proliferation for a given microbe in broth culture

liver: the number of white cells goes up to 5 to 7 times its in controls within 3 hours and remains at this level. At the same time disintegration of lymphoid elements occurred in lymph nodes, spleen and thymus. Its cortex became practically exhausted by the 24th or 48th hour.

In other series of experiments, repeated cultures succeeded in recovering dysentery bacilli from splenic tissue and blood, demonstrating that some of the pathogenes had been able to spread beyond pulmonary tissue. However, we failed to find any bacilli in histological preparations or sections from various organs, or even in mediastinal lymph nodes. However, we did observe the accumulation of granular leucocytes in the distended sinuses of lymph nodes situated about the hilus of the lung. This phenomenon, as well as systemic leucocytosis and disintegration of lymphoid tissue, seem to be due mainly to absorption of antigens of a toxic nature released by the break down of dysentery bacilli in the lungs.

These experimental results have confirmed our earlier conclusion that strains of Sh. dys. 2 do not cause a progressive infectious process in the lung of white mice. Nevertheless, morphological observations have shown that these organisms are able to proliferate in pulmonary tissue: they are unable however, to resist the defence reaction, which sets in soon after inoculation. Such considerations induced us to make additional bacteriological examinations with a view to studying the initial phases of infection. Sixty mice were infected as described above. The results of isolations performed during the first 18 hours, are shown in Fig. 5. It can be seen that they are in perfect harmony with the results of morphological observations. The amount of Shigellae in the lungs was generally higher at 3 hours than at the beginning of the experiments. The slower rate bacterial multiplication in vivo than in vitro is due to the

activity of phagocytes shown to occur in the alveoli at this stage. The number of organisms decreased when a more effective leucocyte reaction was developing in the lung. It should be noted that the rate of bacterial multiplication was suppressed earlier in occasional animals. This seems to correlate to individual intensity of the response as noted in our histological investigations.

3. *Infection induced by Sh. sonnei (S form), Sh. flexneri 2a, Sh. flexneri 6 and Sh. boydii 1*

During the initial phase of these experiments, the appearance of pulmonary tissue was essentially similar to that of mice infected with *Sh. dys. 2* Alveolar phagocytes, however, were still less active in these cases, so that even phagocytosis of single bacilli seemed to be very rare. Accordingly, they could grow almost without hindrance during the first hours. In the case of *Sh. sonnei* infection, for instance, the total number of pathogens was 600 in 100 alveoli two hours after inoculation, *i. e.* 6 to 7 times more than immediately after the inoculation. Some alveoli had become almost completely filled with the pathogens by the 6th to 9th hour (Fig. 6). The animal had not given up its defence, of course. The same pattern of leucocyte response to intrapulmonary inoculation was displayed, no less (in fact, at first even somewhat more vigorously) than in the case of *Sh. dys. 2* infection (Fig. 7). In contrast to macrophages, leucocytes released in to alveoli did their best to engulf a considerable amount of the invaders, as can be seen in Figs. 8. and 9.

Most of the phagocytized organisms were evidently doomed to destruction. We saw them to become gradually smaller, seeming to shrink and, sometimes, to get trapped into vacuoles. On the other hand, we observed also some markedly deformed polymorphonuclear leucocytes that were completely engorged with bacilli. This suggests the possibility of dysentery bacilli being capable of survival and proliferation in certain disintegrating phagocytes. After their destruction, bacilli may escape, presumably to be ingested by other leucocytes. In a number of cases, however, alveoli did not contain an adequate number of leucocytes, so that most of the Sigella bacilli remained free. Such an insufficiency of the leucocyte reaction appeared to be particularly marked in the case of infections with a highly pathogenic *Sh. sonnei* strain. The rate at which the polymorphonuclear leucocytes emerged from the blood vessels was very slow at the onset of these experiments. Still, after 6 to 9 hours, part of the alveoli, filled with serous fluid, contained a considerable amount of granular leucocytes (Fig. 10). While most of the invaders had been phagocytized in such alveoli, there were others (sometimes adjacent ones) which were almost completely devoid of leucocytes and in which bacterial growth was unimpeded. At a later stage, the supply of leucocytes to the lung of most animals was found to cease almost entirely. Leucocytes that had migrated into the alveoli earlier and done their duty there, disintegrated without being replaced. There were comparatively few leucocytes in the capillaries, they were practically absent in the oedematous perivascular spaces and in the bronchi. Only along the margins of the strongly dilated arteries were granular leucocytes sometimes to be seen. (Fig. 11.) In these very animals bacterial multiplication in the pulmonary alveoli was particularly intensive (Figs. 12, 13). It should be noted that the occasionally scattered leucocytes were still in possession of their phagocytic ability and managed to clear some alveoli more or less successfully.

Bacteriological analyses were also made at short intervals in this group of experiments. Their results, illustrated in Fig. 14, conform to morphologic data.

Owing to the initial absence of alveolar phagocytes, bacteria of the *Sh. sonnei* (in contrast to *Sh. dys. 2.*) strain multiplied at the same rate in vivo as in vitro at first. The in-vivo rate became gradually slower along with the development of leucocyte response. (Morphological evidence showed the latter to have reached its peak at 6 to 9 hours following infection.) Reduced rate did not, of course, mean the cessation of growth: at this stage, histological preparations revealed Shigella colonies filling occasional alveoli. The balance between natural reproduction of bacilli and their death by phagocytosis still tended to favour the host. Later, with suppression of the leucocyte reaction, the discrepancy between curves of in-vitro and in-vivo proliferation was reduced once more.

As can be seen from Fig. 14, deficiency of the host's defence manifested itself sooner (as 12 h. as early) in some, and later in other animals. Eventually, however, nearly all of the animals died in this group of experiments. Individual degrees of reactivity made essential differences in results when infection had been caused by *Sh. flexneri 2a, Sh. flexneri 6 and Sh. boydii*, or a less pathologenic strain of *Sh. sonnei*. In some of the mice, the rate at which leucocytes were emerging from the blood vessels seemed to increase steadily, so that hardly any organisms escaped phagocytosis and were found in the exudate-filled alveoli 24 to 48 hours after inoculation. In other animals depression of the leucocytic reaction occurred resulting in

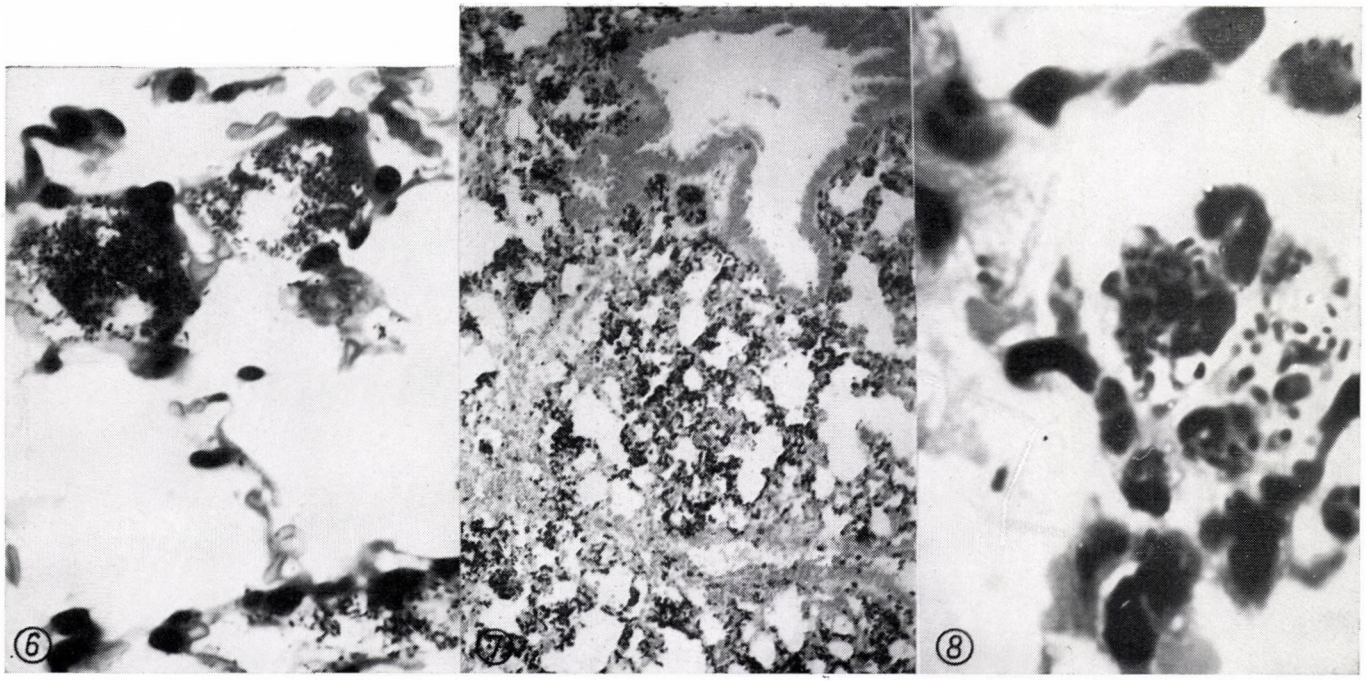

Fig. 6. Sh. sonnei bacilli in pulmonary alveoli, 9 hrs after infection. Azure-eosin. ×900
Fig. 7. Leucocyte reaction in the lung, 3 hrs after inoculation with *Sh. flexneri 2s.* Goldman's dye. ×100
Fig. 8. Leucocytes filled with *Sh. flexneri 2a* in the alveoli, 6 hrs after infection. Azure-eosin. ×2000

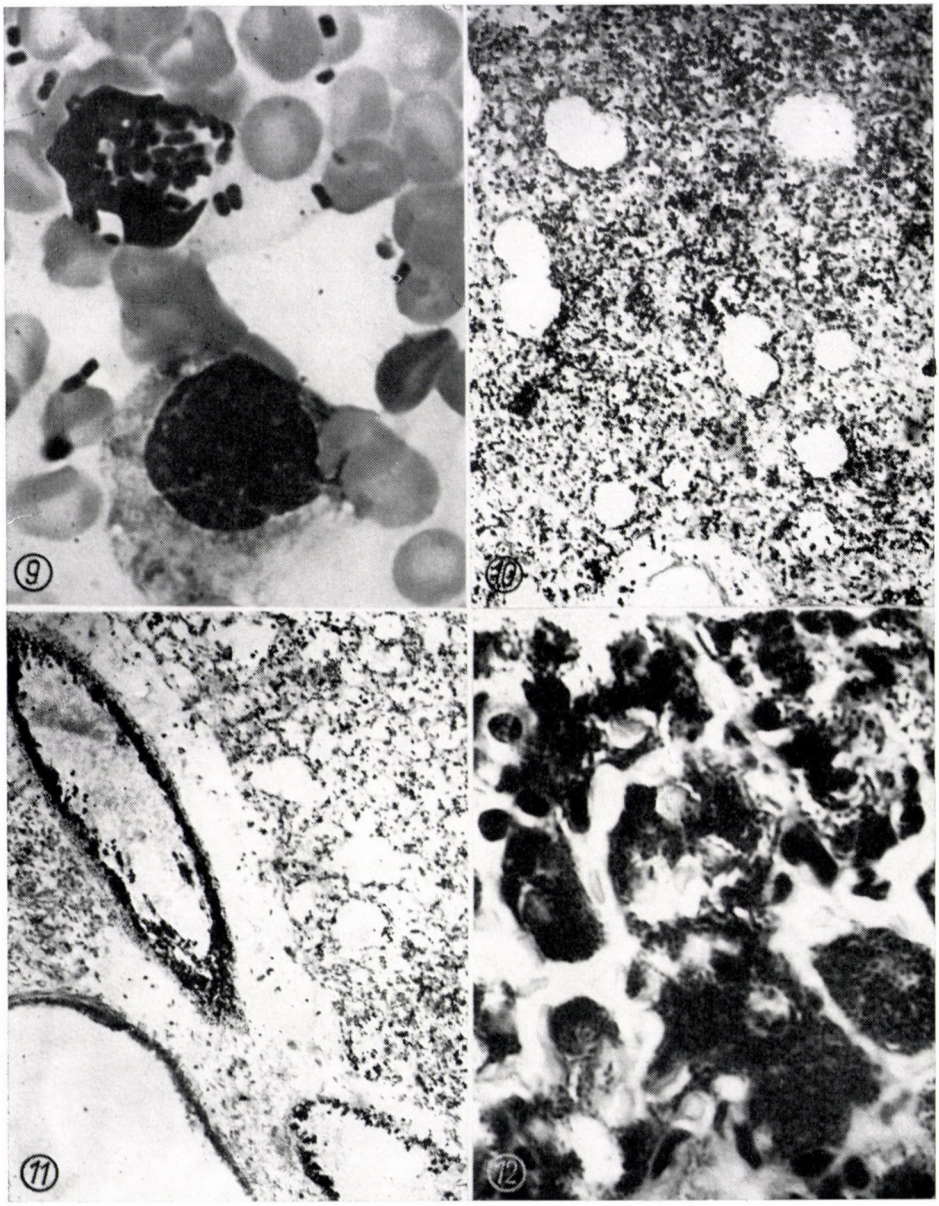

Fig. 9. Phagocytosis of *Sh. sonnei* bacilli by polymorphonuclear leucocytes. Beside, macrophage with uncertain, pale inclusion bodies in the cytoplasm. Romanowsky—Giemsa. ×1700.

Fig. 10. Leucocyte reaction in lung, 9 hrs after inoculation with *Sh. sonnei*. Goldman's stain. ×100

Fig. 11. Some disintegrating leucocytes in the dilated pulmonary arteries, 24 hrs after inoculation with *Sh. sonnei;* also marginally situated leucocytes are visible. Perivascular oedema. Goldman's stain. ×100

Fig. 12. Alveoli filled with *Sh. sonnei*. Note upper middle part of picture: the number of microbes is less on account of the presence of leucocytes. Mouse killed 24 hrs after inoculation. Azure-eosin. ×950

unrestrained microbial proliferation. This will explain the great variations (especially striking in the experiments with *S. flexneri 2a*) in the number of bacteria recovered from the lung 24 hours after infection, as seen in Table 1.

Although the bulk of intranasally introduced Shigella bacilli settle in the pulmonary alveoli, these are not the only site to support growth. Bacterial proliferation has been shown to occur within the epithelial cells of the mucous membranes of bronchi and bronchioles. While this phenomenon was noted in a single animal inoculated with *Sh. dys. 2*, it occurred in the majority of animals killed 2 to 24 hours after having been inoculated with *Sh. flexneri*, *Sh. sonnei*, or *boydii*.

Organisms observed in the cytoplasm of epithelial cells appeared to be perfectly viable, mitotic forms being found among them (Fig. 15). The affected epithelial cells seemed to resist destruction for a long time: their nucleus frequently retained its normal shape even when

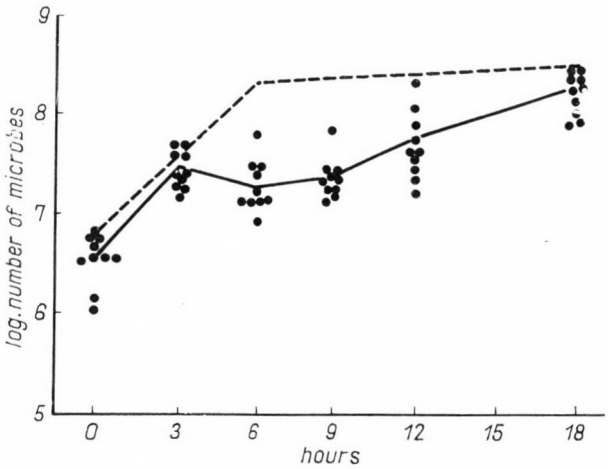

Fig. 13. Great masses of *Sh. sonnei* in Klatsch preparation made from the lung of mouse sacrificed 24 hrs after infection. Romanowsky—Giemsa. ×2200

the cytoplasm had been crowded with growing organisms (Fig. 16). Nevertheless, the ultimate fate of such cells was destruction, they gradually disintegrated and were replaced by polymorphonuclear leucocytes. In some animals, killed at 48 to 72 hours, epithelial regeneration seemed to have taken place over comparatively large areas of the bronchial mucosa, while only a limited number of epithelial cells seemed to have suffered damage in most cases.

Only in cases when very intensive intraalveolar microbial proliferation occurred in dying or sacrificed mice, would growth of Shigella bacilli also be observed in the expended perivascular areas. In such cases, branches of the pulmonary artery were sometimes enclosed by masses formed of Shigella colonies, while the regional pulmonary lymph nodes contained only a few occasional bacteria.

Pulmonary changes caused by Shigella infection were, thus, very variable in this group of experiments. Pneumonia (focal or diffuse) also affected the upper and dorsal parts of the lung most in this group (the left lung was usually involved more; in advanced and lethal cases, however, inflammatory oedema was seen to have spread to all parts. We observed no fibrinous deposits but frequently saw a few erythrocytes in the serous fluid within alveoli. Massiv haemorrhages occurred in the oedematous perivascular areas.

Outside the lung, morphological changes occurred in lymph nodes, spleen and especially in the thymus, where marked breakdown of cellular elements was seen. Leucocyte counts in capillaries of the inner organs, showed that during the first hours leucocytosis was more intensive than in infections with Sh. dys. 2; however, subsequently the number of white cells dropped almost to a normal level even when the local reaction was effective in the lungs.

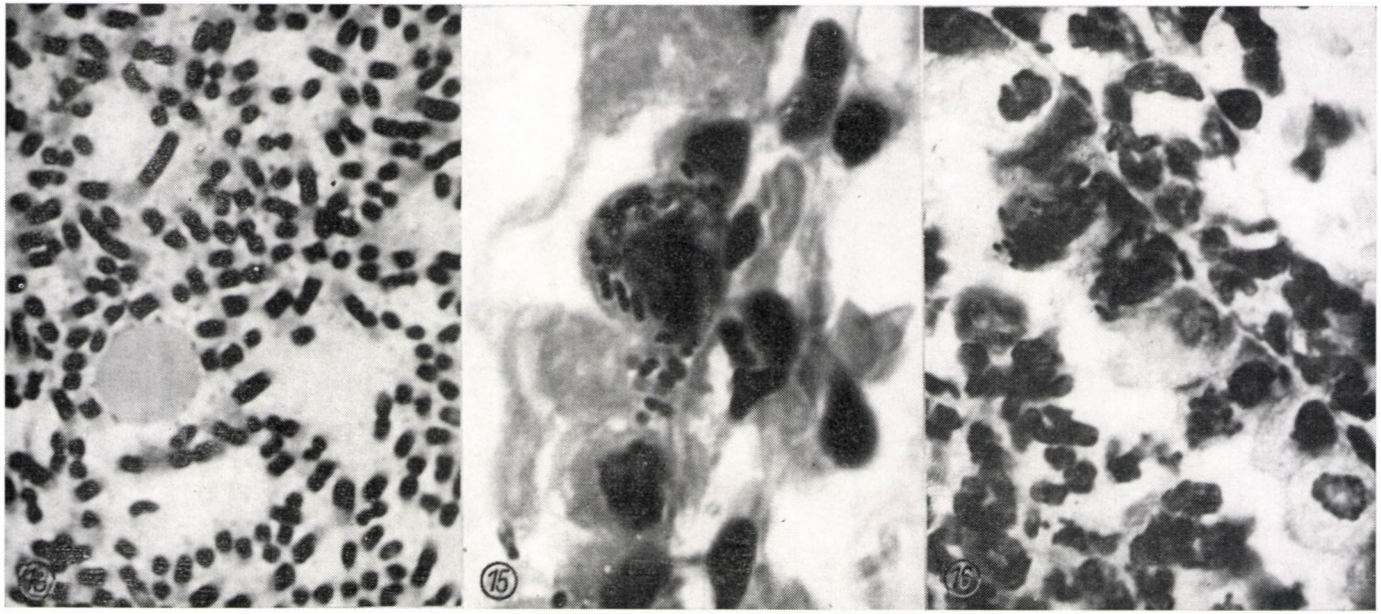

Fig. 14. Sh. sonnei bacilli contained in mouse lunge at different times after infection. For notations see Fig. 5.
Fig. 15. Sh. flexneri 2a in the bronchial epithelium, 6 hrs after inoculation. Azure-eosin. ×2200
Fig. 16. Proliferation of *Sh. sonnei* in epithelial cells with intact nucleus, 9 hrs after infection. Thionine. ×1400

4. *Intranasal infection with killed dysentery bacilli*

It is known that certain micro-organisms (*e. g.* the pathogens of gas gangrene) produce substances opposing the defence reactions of the host, the emigration of leucocytes in particular. The Shigella strains under consideration do not produce exotoxin;* it is possible however, that inactivity of alveolar phagocytes and the frequently observed suppression of leucocyte reaction might be due to an endotoxin released by bacterial disintegration. This assumption seems to be corroborated by the fact that *Sh. dys. 2*, a strain incapable of growing in the lung of mice, is less toxic than *Sh. sonnei* or *flexneri*. True, the latter strains lose their faculty of proliferating in pulmonary tissue after long storage, while retaining their toxicity. It would have been wrong to compare the toxicity of the different Shigella strains on the evidence of our present experiments, since their quantities were subject to changes soon after having been introduced into test animals. Therefore, we studied the effect of equal amounts of intranasally introduced *Sh. dys. 2*, *Sh. sonnei* and *Sh. flexneri* organisms killed by heating at 58 to 60° C.

To mice tolerated inoculation of 5 million (according to the International Standard for Opacity) killed Shigella germs (suspended in broth or saline) quite well. Leucocytes were seen to migrate into the alveoli, accumulating considerably within an hour or two in all experiments of this group. Leucocyte response usually reached its peak at 24 hours, and the alveoli soon became clear of the exudate. Intensity of the reaction seemed to vary in individual animals, but repeated experiments failed to reveal any correlation between the strength of reaction and the type of vaccines.

We proceeded to study vaccines which contained a thousand million germs per 0.05 ccm; *i. e.* the dose administered to the animals. Such a dose was approximately equivalent to maximal amounts of bacilli in the lungs of mice following *Sh. sonnei* inoculation. It proved to be fatal for part of the animals, strains of *Sh. sonnei* and *Sh. flexneri* appearing to be more toxic than those of *Sh. dys 2* (Table 2).

Table 2

Mortality of mice after intranasal administration of a thousand million killed Sh. bacilli

Strain	Percentage mortality after		
	24 hrs	48 hrs	72 hrs
Sh. dys. 2	—	6.0	2.1
Sh. sonnei	—	14.3	27.5
Sh. flexneri 2a	—	17.0	34.0

Local reaction to such quantities of antigen was very strong. Perivascular oedema developed almost immediately and was promptly followed by perivascular haemorrhage, after which polymorphonuclear leucocytes accumulated at a rapid rate in the pulmonary capillaries. They entered some alveoli within 3 hours and focal pneumonia had developed by the 24th hour in the majority of the animals (Fig. 17). Serous exudate was comparatively scarce, but the alveoli were being filled with leucocytes up to the end of the second day. This marked the onset of clearing of pulmonary tissue, a process which often lasted 10 to 20 days. No injury affected epithelium of respiratory passages.

Apart from inevitable individual features of pathology reactions evoked by different types of killed dysentery bacilli also seemed to be essentially similar in these cases. The leucocyte responses to vaccines prepared from *Sh. flexneri* and *Sh. sonnei* were even more pronounced and protracted than to vaccines prepared from an equal number of *Sh. dys. 2* organisms. The most essential observation was that *emigration of leucocytes was not found to have been inhibited in any of the cases.* Even the pulmonary alveoli of animals which had succumbed were densely filled with cellular exudate (Fig. 18).

Since heat-killed bacteria show weaker staining reactions than viable organisms, it was impossible to observe their fate on inoculation into mice. Their phagocytosis by polymorphonuclear leucocytes was highest between the 6th and 24th hour. Alveolar phagocytes also were

* There being no exotoxin-producing *Sh. shigae dys.* in the Soviet Union at present, we have been unable to obtain any fresh cultures of the strain.

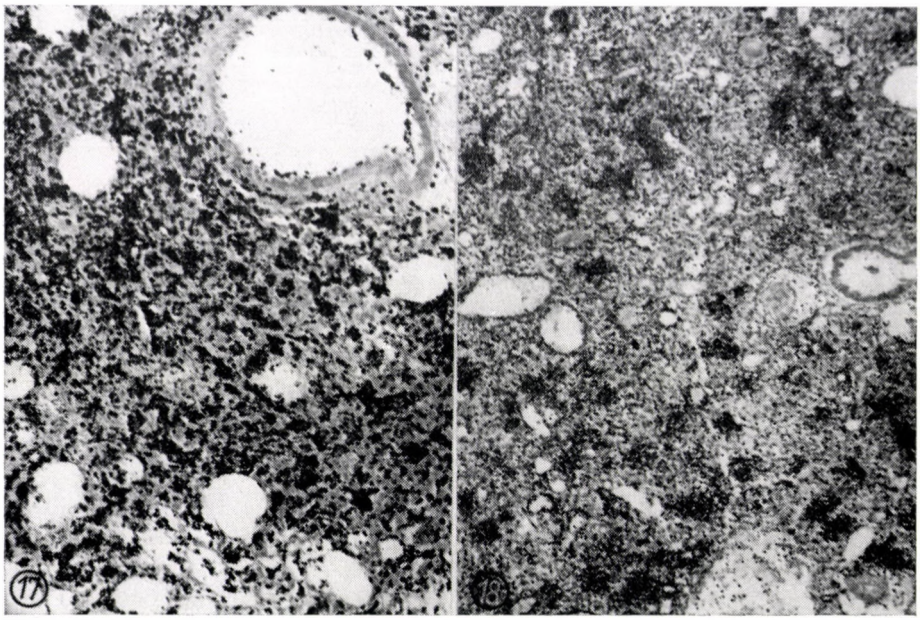

Fig. 17. Accumulation of granular leucocytes in alveoli, 24 hrs after inoculation with a thousand million *Sh. sonnei* bacilli. Goldman's stain. ×100

Fig. 18. Pneumonic foci with leucocytic exudate in lung of mouse which died 48 hrs after infection with a thousand million *Sh. flexneri 2a* bacilli. Goldman's stain. ×50

evidently fairly active in these experiments, although their activity was incomparably weaker than in infections with staphylococci (ILYN, 1957) or H. pertussis (VOINO-YASENETSKY and KHAI, 1960).

Discussion

The results of these investigations provide answers to the problems outlined in the introductory part of this presentation.

Intranasal administration of relatively small amounts of Shigella bacilli to mice induces a pathologic process which, on the evidence of its localization and morphological manifestations, may be regarded as pneumonia. The inflammatory reaction, occurring in the lungs or intestines, in acute dysentery of humans lacks any particular specificity; yet, certain pathologic features, peculiar to the natural disease, as oedema or red blood cell diapedesis, have also been observed in these experiments.

It is a significant fact, that in inoculated animals the activity of dysentery bacilli is fairly strictly limited to pulmonary tissue. As a matter of fact, bacteraemia has been observed in certain instances, but usually of a mild degree and never resulting in the development of metastatic foci or general sepsis. It follows that, by means of quantitative bacteriological assay, the rate of microbial proliferation and disintegration in pulmonary tissue may be

determined with sufficient accuracy at any stage. The particular structure of pulmonary tissue makes it especially suitable for morphologic investigation of processes involving direct interaction between invading organisms, and host.

Enhanced secretion by the bronchial epithelium is one of the earliest reactions to the introduction of Shigella bacilli. This response, however, is of no great significance (although it contributes to clearing the airways of foreign particles), as most of the organisms invade the alveoli, rather than the bronchial tree. Another early reaction is the appearance of a serous fluid in the alveoli. Although the fluid contains plasma proteins apparently involved in defence activity, they exert no effect on the Shigella bacilli. On the contrary: it is the serous fluid which seems to provide a favourable medium for the invaders, and where bacterial proliferation sets in promptly.

Facts obtained in the course of our experiments, are in good harmony with observations made by AVDEYEVA (personal communication) who found that growth of dysentery bacilli could be supported by sera of dysentery patients, as well as by normal human serum.

Pathogens of dysentery are able to survive and multiply in the cytoplasm of epithelial cells lining the trachea and bronchi of mice. Penetration of these cells, lacking any phagocytical potency must be due to active invasion. Intracellular parasitism of this kind plays no important part in the development of infection, following intranasal inoculation. — According to PIÉCHAUD, STURM-RUBINSTEIN and PIÉCHAUD (1958), bacterial proliferation occurs mainly within epithelial cells in cases of dysenteric keratoconjunctivitis. It might seem highly tempting to attribute certain peculiarities in the pathogenesis of human dysentery to intracellular growth of the bacilli. Evidence to this effect, however has not been conclusive yet.

The last of our three questions is the most difficult to answer: indeed, why are some types of Shigellae capable of growing in the lungs of mice, while others are not. Our investigations show all Shigella strains to be potentially capable of proliferation in alveoli and epithelial cells, while not all prove equally able to overcome the host's defence. This biologic property of the bacilli, revealed by animal experimentation, is surely important in the causation of dysentery. Although its underlying nature is not clear, significant features have been disclosed.

A general survey of the pattern of development of the infectious process under experimental conditions makes it evident that the fate of different Shigellae in the lung of mice assumed a different course at the very onset. *Sh. sonnei*, *Sh. flexneri* and *Sh. boydii 1* organisms failed to induce the activity of alveolar phagocytes, known to be effective scavengers of the alveoli. In fact, macrophages were not able to clear alveoli of *Sh. dys. 2* bacteria either, but they destroyed at least part of the invaders and prevented their multiplication to a considerable extent. Thus, the number of organisms present in the

lungs by the time the next phase of the process, *i. e.* leucocyte response, had set in, varied according to the type of the causative agent.

The interaction between the invaders and the emigrating leucocytes proved to be more intricate. While all the strains used in our experiments seemed to be accessible for the leucocytes, the strain *Sh. dys. 2* was phagocytized most effectively. Although most of the members of other strains had been destroyed by the 72nd hour of the experiments, leucocyte activity was not always efficient in suppressing proliferation of bacilli. Leucocytes participating in destruction of the ingested invaders, underwent deterioration, but were replaced by fresh cells. However, replacement of rapidly proliferating bacilli occurred also. Development of the infectious process depended on the outcome of the race between newly appearing leucocytes and Shigellae.

It follows that the more pronounced the inflammatory reaction, *i. e.* the accumulation of leucocytic exudate in the alveoli, the stronger is the resistance of the host. Although some animals succumb to massive pneumonia on account of respiratory failure, death is usually due to the effect of endotoxin, released by phagocytized bacteria. Nevertheless, only the host's response phagocytic activity of leucocytes in the first place, was shown to arrest the progress of the disease.

Experiments with moderately pathogenic bacteria, *e. g. Sh. flexneri 2a*, are best suited for the observation of individual differences in the defence reaction of the host. Results of experiments were far from being uniform. While all animals survived infection with *Sh. dys. 2*, nearly all succumbed to a highly pathogenic strain of *Sh. sonnei*.

It seems probable that certain members of the Shigella group are protected against macrophages on account of their chemical structure, or due to the secretion of some substance. The possibility of active defense of these bacteria against phagocytic activity of the leucocytes is not clear.

It was found in our experiments that the polymorphonuclear leucocytes in pulmonary alveoli retained their phagocytizing faculty even in the gravest processes, as those induced by infection with *Sh. sonnei*; yet, with advancing microbial proliferation, the rate of their emigration from blood vessels decreased, and even ceased altogether in some instances. True, the loss of reactivity in these cases was not principally due to the local effect of substances released by the bacilli, but rather to those systemic disturbances which assumed the form of "bacterial shock". Sometimes, even if the course was favourable and the leucocyte response quite active, there were certain alveoli full of bacteria where leucocytes were absent. This phenomenon of considerable pathogenetic significance may be interpreted in different ways.

It is possible (though unproved) that certain Shigellae secrete some substance ("aggressin") which inhibits the emigration of leucocytes. It seems more probable however, that the invaders being living bacilli, they fail to

induce leucocytes to emigration out of the blood vessels. This does not mean to imply that only dying organisms are accessible to phagocytosis, but rather that the presence of some products of decomposition is necessary as a stimulus for the emigration of leucocyte. This assumption seemed to be confirmed by our experiments with killed Sh. bacilli. Moreover, BIBINOVA (1959) observed pronounced leucocytes reaction in the lungs of rats after the intranasal administration of a complete Sh. flexneri antigen. However, these observations fail to explain why certain Shigellae proved to be more highly pathogenic for the test animals than the others.

In conclusion, another feature, which may be highly significant, has to be noted, can be seen from the curves in Figs. 5 and 14 that Sh. dys 2 and Sh. sonnei cultures grew at different rates in vitro as well as in the lung of mice. In broth cultures, the number of Sh. sonnei bacilli reached its peak as early as in the 6th hour following inoculation, while Sh. dys. 2 bacilli required 12 hours under the same experimental conditions. This fact has to be investigated in particular, since the outcome of the pathologic process in mice was found to be determined in our experiments by the host's response on one hand and by the rate at which phagocytized microbes were replaced.*

Thus in trying to solve the problems essential to the purpose of our investigation, we have been confronted by new ones. It should be emphasized once more that direct interaction between microorganisms and host is displayed in a highly spectacular form in the lungs of mice infected with dysentery bacilli. Although the process is somewhat different from that affecting intestines of human patients, still these experiments in animals furnish some valuable data on the biological properties of Shigella bacilli and on patterns of defence reactions of the host.

* Evidence that rates of proliferation may vary among different members of Shigellae is supported by the investigations of VESELOV (1958) who determined the time required for each division of Sh. flexneri and Sh. sonnei bacilli.

REFERENCES

1. BIBINOVA, L. S., VOINO-YASENETSKAYA, M. K., UGLOVA, T. V., NIKULNIKOVA, N. S.: (1960) Vestn. Acad. Med. Nauk. SSSR. **2**, 34. — 2. BIBINOVA, L. S. OSIPOVA, P. V.: (1958—9) Local effect and morphological changes produced in the lung of white mice by the administration of pertussis and dysentery toxins. Yearbook IEMAMN SSSR. — 3. VESELOV, M. P.: Zh. Microbiol. Epidemiol. Immunobiol: (1958) **4**, 65. — 4. VOINO-YASENETSKY, M. V., VOINO-YASENETSKAYA, M. K.: (1958) Publications of the Pasteur Institute for Epidemiology, Microbiology and Hygiene, **18**, 288. — 5. VOINO-YASENETSKY, M. V., KHAI, L. M.: (1960) Hyg. Epid. Micr. and Immun. (Prague) **4**. — 6. HEKKEL, V. D., BIELAYA, J. A., MELNIK, E. T.: (1957) Z. M. E. I. **7**, 110. — 7. ILYIN, G. I.: Reactive alterations in the lung of white mice after intranasal infection with Staphylococcus aureus. Yearbook IEMAMN SSSR. — 8. MANOLOV, D. G.: (1957) Z. M. E. I. **10**, 94. — 9. SIROKO, I. A.: (1958) Z. M. E. I. **4**, 71. — 10. PIÉCHAUD, M., STURM-RUBINSTEIN, S., PIÉCHAUD, D.: (1958) Ann. Inst. Pasteur, Vol. 94, **3**, 298. — 11. SERÉNY B.: (1955) Acta microbiol. hung. **3**, 293.

Prof. M. V. VOINO-YASENETSKY, Institute Pasteur, Leningrad, SSSR