AN EXPERIMENTAL STUDY ON CELLULAR IMMUNITY IN *PASTEURELLA PESTIS* INFECTION

BY S. S. BHATNAGAR AND D. L. SHRIVASTAVA, *From the Haffkine Institute, Bombay*

(With Plate 7)

In the past there has been much controversy as to whether in host-parasite reactions immunity is conditioned by the humoral or by the cellular mechanism of defence. During recent years explanations for different infective processes have chiefly been forthcoming from the serologist on the basis of various *in vitro* and *in vivo* tests. In spite of advance in our knowledge as to the relative importance of antigenic components of a bacterial cell and their serum counterparts, it can hardly be said that a complete picture of immunity can be drawn from antigen-antibody relationships alone. On the other hand, the latest studies in the field of chemical immunology, with the help of azoprotein antigens by Heidelberger & Kendall (1933, 1934, 1935) and by Sabin (1939) and others, would indicate that tissue cells are responsible not only for cellular immunity but for humoral defence as well, since their cytoplasm is alleged to be identified with the synthesis of circulating antibodies. The fact of the matter would appear to be that both cellular and humoral agents have their respective roles in host resistance; while the study of humoral immunity has progressed because of the availability of technical procedures which can be easily practised, investigations on cellular immunity have been few on account of the paucity of such methods.

Cumulative evidence from a series of studies on *Pasteurella pestis* in relation to active, passive and natural immunity (Bhatnagar, 1940*a*, *b*; Bhatnagar & Kamat, 1946) strongly suggested that humoral factors could only be partly responsible for host resistance against a highly invasive organism of the type of the plague bacillus. Attempts were, therefore, made to obtain information with regard to cellular reactivity to plague infection in animals of which this parasite is a natural pathogen, such as Bombay rats (*Rattus rattus*) and white mice. Certain abnormalities were observed amongst the white cells of blood of plague-infected animals which, when studied by the supravital technique as described by Sabin and her collaborators (Sabin, 1923; Sabin, Doan & Cunningham, 1925*b*; Cunningham, Sabin & Doan, 1925), were found to bear distinct relationship to (*a*) active immunity, (*b*) passive immunity, and (*c*) susceptibility on the part of the experimental animal. The present communication deals with a description of these appearances and discusses the possibility of their application to the study of other bacterial infections.

TECHNIQUE

(*a*) *Experimental animals.* On account of marked differences in their susceptibility to plague infection Bombay rats and Haffkine Institute inbred white mice were employed for this study; while the former are highly immune, the latter are highly susceptible (Sokhey, 1939). It was thus possible to study the cellular changes in an immune and a susceptible animal side by side against a parasite which produces natural infection in both. In earlier experiments animals were infected in batches of fifty, and twice each day two of them sacrificed for cytological study of their heart's blood and smears from the spleen and liver. A constant amount of blood was also cultured at the same time to determine, from the number of colonies, as to how infection was progressing. It was later discovered that it was quite unnecessary to kill the animals, and that cytological study of peripheral blood alone, by repeated partial amputations of the tail of the same animal from day to day, gave information about cellular changes which was more satisfactory.

In view of rhythmic fluctuations in the quality and number of leucocytes during different hours of the day (Sabin, Cunningham, Doan & Kindwall 1925*a*; Shaw 1927), animals were bled every morning and afternoon. Three different observations, besides culture of blood, were made on each sample: (i) study of live cells after supravital staining, (ii) study of fixed smears after staining for peroxidase granules and counterstaining with Wright's stain, (iii) total white cell count. About 1000 white mice and over 500 Bombay rats were employed in this study. To appreciate normal variations in cellular pictures, especially amongst the rats, it was

necessary to experiment initially with a large group of normal animals.

(*b*) *Antiplague sera.* These were prepared in the manner described by one of us in a previous communication (Bhatnagar, 1940*b*). Passive immunization of animals was carried out with pure envelope and pure somatic as well as with whole antiplague serum in order to study blood cytology under all three conditions.

(*c*) *Supravital staining.* Various dilutions of neutral red and janus green were tried for this purpose. It was noticed that a combination of these two dyes was abnormally toxic for the cells in the presence of bacterial infection. The mitochondrial stain had, therefore, to be dropped and neutral red (Grübler) alone used throughout.

A stock solution of 1 % neutral red was prepared by adding 100 mg. of the dye to 10 ml. of neutral absolute alcohol. Slides, cleaned in the usual manner, were roasted in a flame and then polished with jewellers' rouge just before use. To prepare films, the slide was flooded with 1/25 dilution of the stock solution (0·4 ml. being added to 10 ml. of neutral absolute alcohol) and the stain quickly drained off. The slide was then left standing upright till dry. The daily requirement of slides was thus prepared in the morning and stored subsequently in the incubator.

The animal was bled from the tail, and a drop of blood, placed on one end of the prepared slide, drawn with a spreader so as to form a thin smear. A cover-slip was immediately placed over it and the film sealed with a mixture of vaseline and paraffin wax which by experience was found not to melt within a range of temperature of 37–40° C. The slide was then transferred to an incubator at 37° C. and usually examined a few minutes later.

A research binocular microscope (Bausch & Lomb), with eyepiece 10× giving a magnification of 970, was placed inside an improvised cardboard box, the temperature of which was kept at 37–39°C. with the help of two electric bulbs. A maximum and minimum thermometer was employed to regulate the examination of slides at blood heat. Best results were obtained with natural light. Whenever artificial illumination had to be employed it was found necessary to use an appropriate filter to appreciate various shades of colours in different cells.

(*d*) *Fixed smears.* To avoid the possibility of any shrinkage in the size of white cells it was essential to dry blood films immediately after preparation in front of an electric fan. They were then stained in the following manner:

(i) The smear was covered with benzidine solution (0·1 g. benzidine dissolved in 25 ml. of 80 % methyl alcohol and 4 drops of hydrogen peroxide added) for 30–40 sec. followed by dilution of this solution with an equal amount of neutral distilled water and left to act for 3 min. The slide was then drained off and dried.

(ii) Wright's stain (0·1 g. of stain added to 60 ml. of neutral methyl alcohol) was poured over the dried slide and almost immediately diluted with distilled water and left to act for 6–7 min. The slide was then washed under the tap and allowed to dry naturally.

Peroxidase staining was resorted to for the purpose of differentiating between mature and primitive cells, and to determine if increased functional activity, represented by the number of red vacuoles inside cells, was associated with an increase in the number of oxidase granules. No such relationship was, however, observed.

Plague infection and abnormalities in white cells

The characteristics which distinguish the various types of leucocytes in a normal supravital stained blood film have been fully described by Sabin *et al.* (1925*a*). An idea of their pictorial appearance can be obtained by reference to Pl. 7.

Under the stress of bacterial infection certain departures from the normal were observed to take place in the white cells of the peripheral blood stream with respect to (*a*) functional activity, both vacuolar and granular, (*b*) motility, (*c*) cell size, (*d*) general appearance, (*e*) certain disturbances in total number and relative ratio of normal cell types, and (*f*) appearance of abnormal cells.

From a study of these changes it was possible to judge whether the struggle between the host and the parasite was developing in favour of one or the other, and how far any interference in the way of administration of a protective serum was going to influence the ultimate outcome.

Cellular response in relation to active immunity

Bombay rats (*Rattus rattus*) have been known to possess a high degree of resistance to plague infection. With a test-infective dose of $3–4 \times 10^3$ organisms of a virulent culture of *Past. pestis*, given subcutaneously into the abdominal wall, 91 out of 100 animals survived in our last experiment. Animals were infected in this manner and changes in their white blood cells studied from day to day every morning and afternoon.

During the first 48 hr. after infection the polymorphonuclear leucocytes were observed to react to the parasite; there was a great increase in their functional activity represented by a preponderance of both the granules and the vacuoles (see Pl. 7, fig. 2). No changes were so far visible in either the monocytes or the lymphocytes.

At the end of this period, however, it was evident that the polymorphonuclears had spent their force and were faring badly as judged from signs of hypoactivity and degeneration; the movement of some of them was sluggish, others had completely lost their power of locomotion and had become rounded in appearance, presenting a hyaline background with no infiltration of neutral red dye into them or at the most a diffuse pink staining of the entire cell.

The following day a number of abnormal cells had appeared. These have commonly been described as clasmatocytes (Ranvier, 1890), but have also been referred to as histiocytes (Aschoff & Kiyono, 1913), wandering epithelial phagocytes (Sabin *et al.* 1925*a*), and by many other names as morphonuclear cells; they now looked young and fresh like the normal cells. Further examinations after the 7th day did not reveal any departure from the normal cell picture. The general condition of the animal also improved *pari passu*. Since the blood was being examined every morning and afternoon, it must be stated that afternoon examinations gave more satisfactory results than those obtained from the morning specimens. The total and differential cell counts over a period of 7 days in Bombay rats are reproduced in Table 1.

Cellular response in a susceptible animal

Experience over the past few years has always shown that the Haffkine Institute inbred white

Table 1. *Cellular response to plague infection in immune and susceptible animals (differential supravital and total white cell counts)*

Animal ...	Bombay rat (*immune*)						
Time in days after infection...	1	2	3	4	5	6	7
Differential supravital cell count per cent.:							
Lymphocytes	14	8	3	10	12	8	24
Polymorphonuclears	82	86	84	78	50	50	68
Monocytes	4	6	6	4	3	1	2
Clasmatocytes	—	—	7	8	35	41	6
Total white cell count per cu.mm.	11,000	16,000	16,000	32,000	14,000	11,000	10,000

Animal ...	White mice (*susceptible*)					
Time in days after infection...	1	2	3	4	5	6
Differential supravital cell count per cent.:						
Lymphocytes	36	28	26	28	23	22
Polymorphonuclears	46	38	58	50	52	58
Monocytes	18	34	16	22	25	20
Clasmatocytes	—	—	—	—	—	—
Total white cell count per cu.mm.	10,000	12,000	14,000	20,000	18,000	22,000

well (Carrel & Ebeling, 1926). They were easily distinguished by the largeness of their size, 3–4 times the diameter of a red blood cell. Their most characteristic feature, however, was not so much their size as the presence inside them of a large amount of neutral red dye mainly in the form of large globoid masses, the tinctorial appearance of which varied from bright scarlet to deep maroon (Pl. 7, fig. 2). The number of clasmatocytes increased from the 4th to 6th day when they formed a significant percentage of the total number of white cells. On the 7th day, however, there was a sudden drop in their number, and they had almost disappeared from the peripheral blood stream within the next 24 hr. Simultaneously, a change was noted in the general appearance of the polymouse is the most susceptible experimental animal to plague infection. A test-infective dose of $1–2 \times 10^2$ organisms of a virulent culture of *Past. pestis*, suspended in broth and administered subcutaneously into the abdominal wall, usually results in 100 % mortality. The average duration of life of the animal is 5–7 days. Animals were infected in this manner and their blood cytology studied.

A downhill course was indicated from cellular appearances right from the very start. At no stage of infection were clasmatocytes encountered. Although there was a relative increase in the size of cells, some of them being abnormally large, these cells could not be confused with clasmatocytes, since the neutral red dye was invariably absent

from them. The dominating cell was the polymorphonuclear which appeared to form the first and the last line of defence of this animal. There was some increase in the functional activity of polymorphs during the first 48 hr., but it was usually of the granular type; the presence of vacuoles was an exception. An increase was noted in the number of monocytes, some of them being large in size, but they, as a rule, were functionally inactive, there being only a granule or two at the *hof* of the nucleus.

After 48 hr. the locomotion of polymorphonuclear cells began to decrease. With the advance of time more and more of them assumed a rounded appearance with less and less penetration of dye into them. About the 4th day the picture was characteristic. A large majority of the cells were more than average in size, rounded in shape, with almost complete absence of motility and presented a swollen, oedematous, greyish, hyaline appearance which could be said to bear some resemblance to ground glass punctured all over as if it had been eaten by clothes moths. In a small proportion the cell boundary had given way, studding the blood film with masses of smudging (see Pl. 7, fig. 3).

A contrast to the picture described was presented by the examination of a fixed smear. This showed evidence of marked leucocytosis with signs of activity on the part of haemopoietic system in the shape of reticulocytes and primitive cells of myelocytic origin with oxidase granules. According to common belief such a picture would indicate that the animal was putting up a good resistance. The 'ground-glass' and 'moth-eaten' appearance of the cells in a supravital film, however, always connoted a very grave prognosis, and the case invariably ended fatally. Table 1 gives an idea of the total cell counts and the differential ratios of various cell types at different stages of infection in a white mouse over a period of 5 days.

Cellular response in relation to passive immunity

An antiplague serum has been shown to be possessed of two different components: the envelope and the somatic (Schütze, 1932; Bhatnagar, 1940*b*). While the envelope antibody is highly antiparasitic, the somatic antibody is devoid of any protective value (Schütze, 1934). It was, therefore, considered desirable to study the cellular reactions of animals given monospecific—envelope and somatic—as well as the whole antiplague serum. In preference to rats, white mice were considered more suitable, since results could be obtained uninfluenced by an unknown factor in the form of any acquired immunity such as occurs in rats. Cellular changes were studied for a period of 30 days, since it has been our experience that any plague-infected mice surviving longer could be said to have resisted the infection successfully. The test-infective dose—$1–2 \times 10^2$ organisms of a virulent culture of *Past. pestis*—and the antiplague serum were injected simultaneously under the skin of the opposite flanks of the abdomen of the animal in batches of 100 animals at a time. On account of economy of space only the broad facts from a large series of experiments are represented here.

Envelope serum and cellular response

Taking advantage of previous knowledge on passive immunization, batches of white mice were injected with 0·3, 0·15 and 0·05 c.c. of pure envelope serum, the agglutinin titre of which was 500–1000. With the maximum dose (0·3 c.c.) it had been possible to save 80–100 % of animals against the test-infective dose employed; with smaller amounts of serum (0·15 and 0·05 c.c.) the life of the animal was prolonged from 5–7 days, in the case of unprotected animals, to 14–21 days before it eventually succumbed to plague infection. Cellular reactions could thus be studied amongst partially protected and more or less absolutely protected animals.

Waves of cellular reactivity exhibiting a certain periodicity characterized the blood cytology of these animals. During the first 2 days there was marked functional activity represented by large-sized bright scarlet vacuoles and granules inside the cells. This was accompanied by the appearance of clasmatocytes in the peripheral blood stream. While the intensity of cellular reaction was proportional to the potency and dosage of envelope serum, the presence of clasmatocytes was dependent not only on these two factors but also on the route of administration of serum; in the few experiments carried out, the same amount of serum given intravenously evoked a clasmatocytic response, whereas it failed by the subcutaneous route.

This activity, however, did not last long and had by the end of 72 hr. disappeared entirely, to be followed by a change in the relative proportion of various types of cells. A significant increase in the number of monocytes at the expense of polymorphonuclears and lymphocytes was noticed, although the total cell count remained within the normal range (see Table 2).

On the 7th–8th day there was another wave of activity. The number of vacuoles and granules inside the monocytes and polymorphs had increased suddenly and the clasmatocytes reappeared. This phase, however, did not last more than 24 hr. and was quantitatively of a lower order than that observed within the first 2 days of infection.

Depending upon the potency and the dosage of

serum, the animals started dying from the 10th day onwards. In the serum-protected mice, even with as small a dose as 0·05 c.c., it was unusual for any mortality to occur before the end of this period.

In animals which succumbed to infection, there was, on an average about 24 hr. before death, a sudden change in the blood picture. The number of monocytes was seen to go down and the total number of leucocytes to go up along with a marked relative increase in the number of polymorphonuclears. A great majority of these cells, however, had assumed a more or less rounded shape with practically no locomotion and almost complete absence of functional activity. Different stages of degeneration were on the up-grade. This state of affairs continued till the animal died.

In animals which survived the second week of infection, there was a third wave of functional activity about the 15th day. It was, however, unusual for the clasmatocytes to put in another appearance. On the other hand, the high level of monocytes was maintained throughout the period of 30 days in animals which successfully resisted the plague infection. This fact was of great prognostic significance, since any tendency towards a fatal termination was immediately detected by reduction in the number of monocytes and preponderance of functionally inactive polymorphonuclears with simultaneous increase in the total number of leucocytes. A certain percentage of degenerated cells was seen to be present throughout the course of infection.

The total and differential cell counts from animals which survived for different periods under the protective influence of envelope antibody are given in Table 2.

Table 2. *Cellular response in white mice inoculated with test virus + pure envelope antiplague serum (differential supravital and total white cell counts)*

Average duration of life of experimental animal	14 days					21 days					
Dosage of serum... ...	0·05 c.c.					0·15 c.c.					
Time in days after inoculation ...	1	4	8	11	14	1	4	8	11	14	21
Differential supravital cell count per cent.:											
Lymphocytes	20	16	12	14	15	16	16	19	20	18	10
Polymorphonuclears	60	50	43	45	79	61	49	44	46	44	88
Monocytes	12	34	40	41	6	13	36	29	34	38	2
Clasmatocytes	8	—	5	—	—	10	—	8	—	—	—
Total white cell count per cu.mm.	8,000	10,000	14,000	19,000	35,000	7,000	11,000	12,000	14,000	17,000	30,000

Average duration of life of experimental animal	Survival over 30 days						
Dosage of serum	0·3 c.c.						
Time in days after inoculation ...	1	4	8	11	14	21	30
Differential supravital cell count per cent.:							
Lymphocytes	18	16	19	15	22	20	24
Polymorphonuclears	51	42	37	38	43	43	37
Monocytes	16	42	35	47	35	35	39
Clasmatocytes	15	—	9	—	—	—	—
Total white cell count per cu.mm.	8,000	14,000	22,000	15,000	12,000	10,000	10,000

Somatic serum and cellular response

Since it was known from previous experiments that the somatic antibody does not contribute to the protective value of an antiplague serum, only the maximum dose of somatic serum—0·3 c.c. agglutinin titre 20,000—was given to all the animals. The cellular picture was entirely different to that in the case of envelope serum. It was, nevertheless, characteristic in its own way.

Within 24 hr. a tremendous increase in the number of leucocytes (30,000–40,000) was observed. This was kept up for the next 24–48 hr. There was

https://doi.org/10.1017/S0022172400019094 Published online by Cambridge University Press

then a sudden drop to be followed by another rise continuing till the animal died. The functional activity was characteristically depressed throughout the course of infection; it compared unfavourably even with that seen in control mice. Most of the cells were round in shape and refractile in appearance, their size being so small that it was difficult to distinguish the polymorphonuclear cells from the lymphocytes with consequent abnormal discrepancy between the counts of supravital and that of fixed smears. All stages of degeneration were present from the second day onwards, the animal, in a majority of cases, dying even earlier than the controls.

Cellular response to antiplague sera

From a description of the cellular changes in the presence of a pure envelope and pure somatic serum, it can be inferred that the cellular reactions of an animal under the influence of an antiplague serum, in which both these antibodies are present together, will be the resultant of two diametrically opposed factors, one of which is trying to stimulate the power of resistance of the host while the other at the same time is depressing it. The end result will, therefore, depend on how far the somatic component is able to annul the protective action of the envelope antibody. This has been our experience with all the antiplague sera, having different titres of envelope and somatic antibodies, which we have employed for the study of blood cytology, and it has afforded an explanation as to why in earlier experiments on passive immunization (Bhatnagar & Kamat, 1946) it was not possible to correlate quantitatively the potency of an antiplague serum with the agglutinin titre of its envelope antibody content.

DISCUSSION

The evidence presented in this communication suggests that in a bacterial infection immunological inferences of great value can be drawn from the supravital study of cellular changes in the peripheral blood stream. The present investigation developed as a side issue to an important problem in plague serology, namely, the difficulty of assessing the potency of antiplague sera by biological methods and the correlation of such tests with serological findings. The deleterious effect of the somatic component of an antiplague serum, so clearly brought out by the study of cellular response to somatic antibody, is in conformity with the results of earlier animal experiments described separately (Bhatnagar & Kamat, 1946). Thus it provides an important observation to be applied to problems of plague prophylaxis and plague-serum therapy.

In view of the fact that different abnormalities of the cell pictures and the different behaviours of individual cell types are intimately related to susceptibility on the one hand and immunity, both active and passive, on the other, it would appear that these cellular variations are an index of the biological activity of the organism, in that they represent its capacity to mobilize cellular defences under pathological conditions. As such their study in relation to other bacterial antigens and antibodies, by the simple method here described, is likely to throw light on the complex mechanism of immunity from the cellular as well as the humoral points of view.

The identification of the role of various cell types in a bacterial infection is related to the basic studies of Metchnikoff on macrophages and microphages. Recent investigations by Gay and his associates (Gay, 1935) on experimental streptococcal empyema in rabbits would, however, indicate that Metchnikoff attached undue importance to microphages (polymorphonuclear cells), while the primary role of defence lay with the cells of the reticulo-endothelial system—the clasmatocytes (macrophages) and the monocytes. The observations recorded here would lend support to the latter point of view. Both in experiments on acquired and passive immunity the detection of clasmatocytes in the peripheral blood was found to be an objective observation of great prognostic value. The association of monocytes with passive protection was well marked; their maintenance at high level connoted protection, while a significant diminution in their number heralded a fatal termination. On the other hand, so far as the polymorphonuclear cells were concerned a large increase in their total number was seen to precede the death of the animal both in the case of serum-treated and non-treated white mice. It would, therefore, appear that the protective value of a leucocytosis of this cell type is practically nil.

In the early stages of infection the presence of bacteria within the granulocytes and their absence from other cell types was suspected. Although the culture of such samples of blood were negative, the overwhelming presence of plague bacilli inside them in the later stages of infection would indicate that the function assigned by Metchnikoff to microphages, namely, that they are the porters of organisms, is very likely true. A similar observation in the case of *Streptococcus* infection by Gay & Morrison (1923) is of interest.

It is to be emphasized that if cytological investigations are to be applied to the problem of infection and immunity they must include not only the morphological analysis of various cell types but

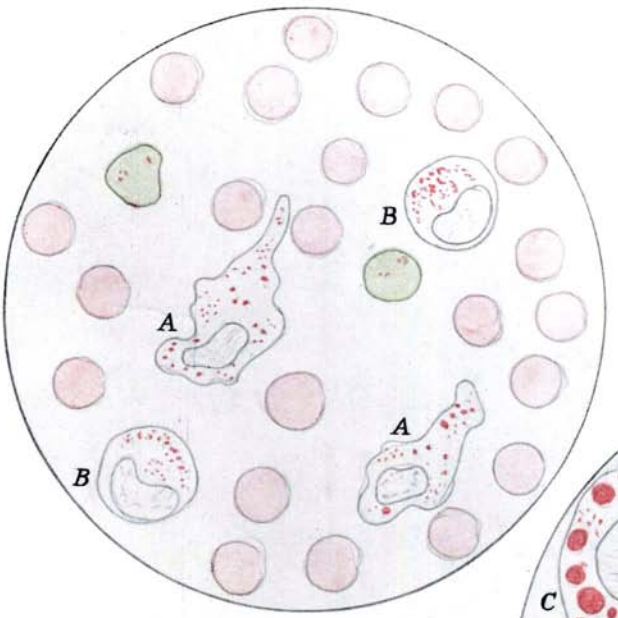

Fig. 1.

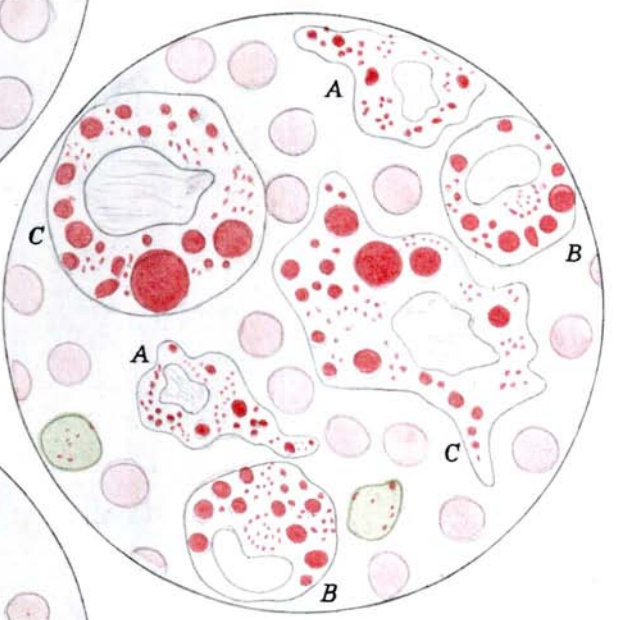

Fig. 2.

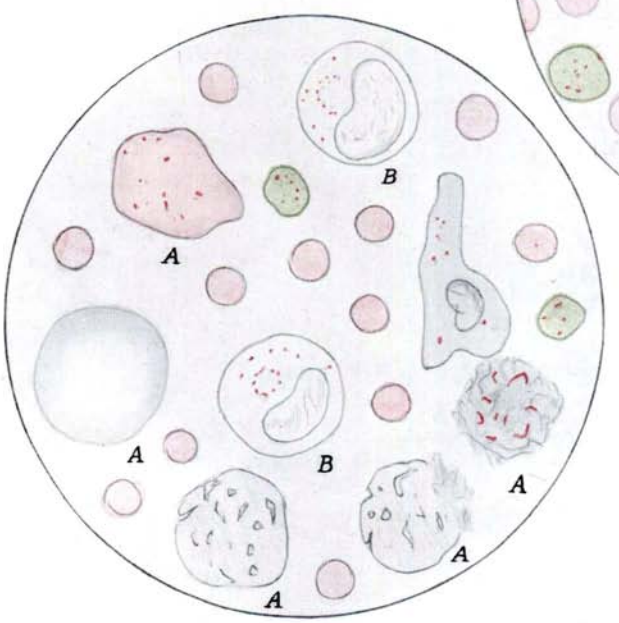

Fig. 3.

A. Granulocytes.
B. Monocytes.
C. Clasmatocytes.

Leucocytes in supravital-stained films of blood.

Fig. 1. In normal blood (rat).
Fig. 2. In blood within 48 hours of infection with *Past. pestis* (rat).
Fig. 3. In blood about the 4th day after infection (mouse).

https://doi.org/10.1017/S0022172400019094 Published online by Cambridge University Press

also the more important study of physiological properties of these protoplasmic structures in a live state by such methods as are at our disposal. The penetration of a non-toxic dye, such as neutral red, into the cell protoplasm and its distribution in various patterns is recognized to be connected with cell metabolism (Carrel & Ebeling, 1926). The neutral red vacuoles characterize a cell in a state of functional activity and represent its organ for intracellular digestion. An increase or a decrease in their size is associated with hyper- or hypo-activity on the part of the organism as a whole. Our experiments with a living particulate material fully confirm this view. Both in the case of immunity and susceptibility the balance in favour of the host or parasite could be judged by an enhancement or a diminution in the amount of the neutral red material taken up by the cell. The various degrees of functional activity encountered by us, from fully fledged vacuolar formation to diffuse pink staining, would thus represent a pathological adaptation of one of the innate physiological properties of cell protoplasm, and since they are shared by almost all the cell types they can be taken to be a manifestation of common responsibility on the part of cellular defence mechanism for the purpose of community survival. The individual variations amongst the clasmatocytes, the monocytes and the polymorphonuclear cells in this respect are an indication of their different potential phagocytic capacities in relation to the role which the reticular and the endothelial derivatives of the reticulo-endothelial system of cells are called upon to play in host-parasite relationships in conjunction with the microphages.

SUMMARY

1. Animals susceptible and naturally immune to plague—Bombay rats and white mice—were infected with *Past. pestis* and supravital study of white blood cells from the peripheral blood stream carried out. Similar studies were made on white mice injected with (*a*) pure envelope serum, (*b*) pure somatic serum, and (*c*) whole antiplague serum.

2. Different experimental conditions produced different cell pictures with different behaviours of individual cell types, especially the polymorphonuclears, the monocytes and the clasmatocytes. These abnormalities were found to bear distinctive relationship to (*a*) active immunity, (*b*) passive immunity, and (*c*) susceptibility on the part of the experimental animal.

3. The value of immunological inferences from this study in relation to plague-serum therapy and plague prophylaxis has been emphasized.

4. The possibility of a better understanding of host-parasite relationships from similar studies in other bacterial infections has been pointed out.

Our thanks are due to Lt.-Col. S. S. Sokhey, I.M.S., Director Haffkine Institute, for his interest in this investigation.

REFERENCES

ASCHOFF, L. & KIYONO, K. (1913). *Folia haemat., Lpz.*, **15**, 383.
BHATNAGAR, S. S. (1940*a*). *Indian J. Med. Res.* **28**, 1.
BHATNAGAR, S. S. (1940*b*). *Indian J. Med. Res.* **28**, 16.
BHATNAGAR, S. S. & KAMAT, G. K. (1946). *Indian J. Med. Res.* (in the Press).
CARREL, A. & EBELING, A. H. (1926). *J. Exp. Med.* **43**, 461.
CUNNINGHAM, R. S., SABIN, F. R. & DOAN, C. A. (1925). *Contr. Embryol. Carneg. Instn*, **16**, 227.
GAY, F. P. (1935). *Agents of Disease and Host Resistance*, p. 296.
GAY, F. P. & MORRISON, L. F. (1923). *J. Infect. Dis.* **33**, 338.
HEIDELBERGER, M. & KENDALL, F. P. (1933). *J. Infect. Dis.* **58**, 137.
HEIDELBERGER, M. & KENDALL, F. P. (1934). *J. Infect. Dis.* **59**, 519.
HEIDELBERGER, M. & KENDALL, F. P. (1935). *J. Infect. Dis.* **62**, 467.
RANVIER, L. (1890). *C.R. Acad. Sci., Paris*, **110**, 165.
SABIN, F. R. (1923). *Johns Hopk. Hosp. Bull.* **34**, 27.
SABIN, F. R. (1939). *J. Exp. Med.* **70**, 67.
SABIN, F. R., CUNNINGHAM, R. S., DOAN, C. A. & KINDWALL, J. A. (1925*a*). *Johns Hopk. Hosp. Bull.* **37**, 14.
SABIN, F. R., DOAN, C. A. & CUNNINGHAM, R. S. (1925*b*). *Contr. Embryol. Carneg. Instn*, **16**, 125.
SCHÜTZE, H. (1932). *Brit. J. Exp. Path.* **13**, 284.
SCHÜTZE, H. (1934). *Brit. J. Exp. Path.* **15**, 200.
SHAW, A. F. B. (1927). *J. Path. Bact.* **30**, 1.
SOKHEY, S. S. (1939). *Indian J. Med. Res.* **27**, 341.

(*MS. received for publication* 9. VII. 1945.—Ed.)