EXPERIENTIA

Vol. XXII – Fasc. 1 Pag. 1–64 15. I. 1966

Phagocytosis and Immunity

By D. Rowley

Department of Microbiology, University of Adelaide (Australia) and
Department of Biochemistry, University of Lausanne (Switzerland)

The Central Role of Phagocytosis in Immune Reactions*

In the following pages I hope to draw your attention to the important and possibly decisive contribution made by phagocytic cells to the overall immune response. Having thus persuaded you that macrophages and other phagocytes are worth studying, we will examine in some detail the interactions between macrophages and particulate antigens such as bacteria. Finally we will apply our considerations to the extremely narrow field of one particular bacterial infection of mice – that known as mouse typhoid – which may serve as a model for many other host-parasite conflicts.

Those of you who are familiar with current immunological thought will realise that there is, at the moment, a great debate concerning theories of antibody production. The issue is the same as that between Darwinian genetics and Lamarckism; the same as that which faced microbiologists a decade or two ago in trying to understand the origin of drug resistant strains of bacteria. The issue is that of adaptation versus mutation and selection. In other words, whether antigens can change cells phenotypically so that they produce various antibodies or whether antigens merely act to select those cells which are, by virtue of their *genotype*, already producing a certain restricted number of antibodies.

I am not competent to try to resolve this question for you, nor for that matter do I believe the answer to be very important. The useful purpose of these immunological polemics lies in the new knowledge which we gain in struggling to achieve a decisive answer – a goal which may never be reached. My objective is to remind you that whatever the exact mechanism of antibody production, the act of phagocytosis seems certain to play a large and perhaps crucial part in the initiation of the antibody-forming process.

If we examine the immune response, without at this time giving much attention to detail, we can say with certainty that if antigenic material is introduced into animals by any route, it will soon be found localized inside cells, such as macrophages and other components of the reticulo-endothelial system. Yet when antibody appears, some days later, it comes mainly from cells such as lymphocytes and plasma cells which are clearly different from those which contain antigen. As the production of antibody is in this case obviously related specifically to the introduction of antigen, there are in the broad sense limited numbers of possibilities to account for these facts. I can only think of three. It is possible firstly that the observed removal of the greater part of the antigen by cells of the reticulo-endothelial system is irrelevant to the whole antibody forming process; and that a very minor, undetected quantity of antigen has reacted directly with the antibody-forming cells and produced a direct stimulation without the intervention or participation of other phagocytic cells at all. This seems to me rather unlikely in view of the fact that the same macrophage and reticulo-endothelial scavenging system which takes up antigen in mammals, was developed early in phylogeny and occurs in the most primitive metazoa. In these animals, as will be seen later, this system has all the attributes necessary for an efficient self/non-self recognition mechanism, which has been emphasized to be the foundation of the immune response. There are other bits of direct evidence, none of them entirely satisfying, e.g. the pyroninophylic antibody-producing cells appear in the same locations as the macrophages which contain antigen, and the negative finding that antibody synthesis cannot be provoked in vitro by antigen plus lymphocytes alone. The alternative possibilities both involve macrophages. Either macrophages or some other phagocytic cells change into plasma cells after digesting the antigen, and this possibility has few supporters, or finally,

* This paper represents the *first* of three Warner-Lambert lectures given at Rutgers University in October 1964.

information about the antigen may be transferred from the macrophages to the antibody forming cell. This information must contain a highly specific element related to the chemical structure of the antigen and it must also say, in nautical language: full steam ahead!

You will all know about the work of FISHMAN[1] which points in this latter direction. In effect he finds that for antibody production to occur in vitro a mixture of lymphocytes and macrophages is necessary – neither will work alone. The hypothetical message which it seems must pass from macrophage to lymphocyte can be destroyed or inhibited by ribonuclease. There are still a few snags in this story but you will see that if all is well this constitutes extremely powerful evidence for the transfer of information between the antigen-binding macrophage and the antibody-producing lymphocyte. The ability of macrophages to decide that a given material is foreign or antigenic and the nature of the message passed to the lymphocyte can be seen to be very fundamental questions of molecular biology. This is my first and main reason for soliciting your attention on behalf of the poor neglected macrophage.

How can macrophages determine that a substance is foreign or not? When phagocytic events have been studied under as well-defined conditions as are available, we see that for the process to be efficient, the particles or antigen must have reacted with some serum factor, usually antibody. This requirement for a serum recognition factor has all the specificity which is found in other immunological phenomena. If there are antibody-reactive molecules present to combine with the particle, this will be phagocytosed: the recognition is provided by the possession of antibody. Even where the particle is composed of so-called 'inert' material there is evidence of this requirement. Several workers have confirmed the observations of STRAUSS and STETSON[2] that uptake of polystyrene particles by leucocytes is dependent on the presence of serum globulins which can be adsorbed out by polystyrene as though it were an antibody.

When we look further at the properties of reticulo-endothelial cells we find that they are rather unique in two ways. They are very well endowed with the hydrolytic enzymes necessary for digestive function, and in addition they show a remarkable capacity for rapid multiplication and increased functional activity following phagocytosis. Cytochemical staining shows that Kuppfer cells of the liver possess high levels of acid phosphatase by comparison with the other cells around them and it has frequently been noticed that during chronic infections these phagocytic cells contain even greater amounts of the enzyme. Working with mouse peritoneal macrophages we and other workers have found high levels of acid phosphatase, β-succin oxidase, and cathepsin. COHN and HIRSCH[3] have shown that these hydrolytic enzymes are associated with cytoplasmic granules which can be seen to burst following phagocytosis, liberating their enzymes into a digestive vacuole. In our laboratory we have found that injection of many particulate colloids into mice, but most effectively of lipopolysaccharide, causes a rapid increase both in the numbers of granules per macrophage and in the total content of various enzymes within these phagocytic cells. We have suggested that this may be an important component of cellular resistance to infection[4]. In any event we can see that the performance of their function of phagocytosis causes macrophages to change in such a way that they can fulfil this function even more efficiently. In addition to such intracellular modifications there are concurrent changes in the total number of these cells. It is now clear that a considerable component of the increased reticulo-endothelial system ability which occurs within 24 h of a suitable stimulus, is due to the multiplication of pre-existing phagocytic cells. KELLY et al.[5] showed recently that the very Kuppfer cells which had taken up particles, were the ones which divided to yield more phagocytic cells. It seems to me that this is probably an example of a general phenomenon produced by the combination of antigen and antibody at the surface of cells. There are examples of this with lymphocytes, eosinophils, peripheral leucocytes, and peritoneal macrophages. MACKANESS[6] has shown that mice, sensitized to an antigen, when injected intraperitoneally some time later with a second shot of the same antigen will respond with a rapid multiplication of peritoneal macrophages. Within 24 h of the second injection as many as 25% of the macrophages may show mitotic figures, and a pulse of H^3 thymidine may label up to 60% of the peritoneal cell population. The stimulus appears to be specific for the sensitizing antigen. Since the work of FORBES and MACKANESS[7] was done in vivo one could not be certain that the new dividing cells arose from pre-existing macrophages. Dr. LEUCHTENBERGER and I have extended this work by giving the second injection of antigen in vitro to tissue cultures of sensitized mouse peritoneal macrophages[8]. When the DNA content of individual macrophages was measured by a microspectrophotometric method, it appeared that there was a shift to a higher DNA content in these cells following in vitro stimulation. In other words it seems probable that contact of antigen with sensitized macrophages can induce them

[1] M. FISHMAN, J. exp. Med. *114*, 837 (1961).
[2] B. S. STRAUSS and C. A. STETSON JR., J. exp. Med. *112*, 653 (1960).
[3] Z. A. COHN and J. G. HIRSCH, J. exp. Med. *112*, 1015 (1960).
[4] IEVA AUZINS and D. ROWLEY, Aust. J. exp. Biol. med. Sci. *40*, 283 (1962).
[5] L. S. KELLY, B. A. BROWN, and E. L. DOBSON, Proc. Soc. exp. Biol. Med. *110*, 555 (1962).
[6] G. B. MACKANESS, J. exp. Med. *116*, 381 (1962).
[7] I. J. FORBES and G. B. MACKANESS, Lancet *1963 ii*, 1203.
[8] D. ROWLEY and C. LEUCHTENBERGER, Lancet *1964 ii*, 734.

to multiply. Both the increased functional ability and the increase in numbers provide the animals with an excellent physiological response to immunological stress.

As with the physiological responses to various stimuli, there must also in this case be a limiting or controlling constraint put normally on the immune response. If this were not so, then continued contact with antigen would result in a neoplastic division of cells leading to an overproduction of antibody, as appears to happen in myelomatosis. It seems likely that with increasing amounts of antibody, new mechanisms are brought into play which act so as to limit the immune response. There are many examples of this. Injection of soluble antigen/antibody complexes formed in antibody excess gives little or no antibody response. FITCH and ROWLEY[9] have shown in rats that prior injection of passive antibody may completely inhibit the active antibody response to foreign red cells. Perhaps the most impressive demonstration that such examples are not unimportant laboratory phenomena, is to be found by examining the influence of ABO blood groups on the incidence of hemolytic disease of the new-born. As you know, in the case of a Rh-negative mother with a Rh-positive foetus, there is a chance that Rh-positive red cells from the foetus will cross the placenta into the circulation of the mother, who may then form anti-Rh globulins. If these occur to a sufficiently high titre they may cross into the child's circulation about the time of birth and cause hemolytic disease. The incidence of this disease, which is quite rare, is much higher in women whose major blood group is the same as that of the foetus. If the mother possesses antibody to the major ABO components of the Rh-positive foetal cells, she tends to form less anti-Rh antibody than the mother whose major group is compatible with her foetus. It is believed that this is due to the rapid elimination and degradation of foetal red cells by a mother who possesses isohemagglutinating antibody against the major parts of the foetal cells. Since the foetal cells are then rapidly eliminated, there is less opportunity for them to exert their antigenic stimulus.

Another possible control mechanism to exert a brake on excessive antibody synthesis which should be considered, is the cell damage which often occurs when antigen/antibody reactions take place at cell surfaces in the presence of complement. At higher concentrations of antibody, the complexes formed with new antigen will tend towards the type antigen/antibody$_2$ which has the greatest C^1-fixing ability and presumably therefore the greatest cytotoxic effect. It is possible that when the antibody concentration reaches a certain level, sufficient of it will adhere to lymphocytes and macrophages and that new antigen will react with these so as to destroy them by the well-known cytolytic effect of antibody + C^1. My only real evidence is that provided by the work of UHR and BAUMAN[10], who studied the effect of pre-existing antibody on the antibody response to diphtheria toxoid. The significant finding is that even when given *5 days after* the antigen, at a time when antibody synthesis was certainly going on, the passive administration of antibody could depress further antibody production. This obviously implies the inhibition of a pre-existing antibody synthetic mechanism and may be a good model for the second controlling path suggested. To summarize at this point, we see that contact of antigen with tissues in the presence of very low levels of antibody is followed by a rapid increase in both numbers and functional ability of the cells associated with the immune response, and this leads to further antibody production. On the other hand, at increasingly higher antibody levels, controls begin to operate in such a way as to limit further antibody release.

There is an apparent difficulty in this suggested scheme which I have so far avoided, this is, if reactive proteins are necessary for uptake of antigen by macrophages in order to stimulate further antibody production, what induces the formation of these early antibodies? It would obviously imply that somewhere along the production line of development the animal formed antibodies (more or less specific in character) in the absence of antigenic stimuli. Once this initial antibody was formed, of course, an antigenic stimulus could work in the way I have suggested. This, after all, is not very different (in degree of difficulty to visualize) from the clonal ideas where it is proposed that separate clones of antibody-forming cells arise, by mutation, in the absence of antigenic stimulus. This is where we reach the essence of the question of Darwinism versus adaptation. It really comes down to this – does the fertilized egg contain, whilst still in the single cell stage, the DNA and RNA templates to provide the information for antibody synthesis of all kinds? Or do these DNA templates arise by mutation at an extraordinary rapid rate, so that by the time of birth approximately 100 cell generations later, there are many hundreds of different antibody forming clones which have arisen by mutation?

There is some evidence that the former is possible – using sea urchin's eggs GROSS et al.[11] have found that in the presence of actinomycin D which inhibits RNA synthesis, the eggs continue to synthesize protein in the normal way. They conclude with admirable conservatism that if RNA templates are necessary for protein synthesis, then the egg must be provided with a little store of this RNA. It could be the same with regard to antibody synthesis.

[9] D. A. ROWLEY and F. W. FITCH, J. exp. Med. *120*, 987 (1964).
[10] J. W. UHR and J. B. BAUMANN, J. exp. Med. *113*, 935 (1961).
[11] P. R. GROSS, L. I. MALKIN, and W. A. MOYER, Proc. nat. Acad. Sci. USA *51*, 407 (1964).

The conceptual difficulties in the idea of pre-existing antibody as a requirement for antibody synthesis would be diminished if the natural pre-existing antibody was much less specific than the acquired kind. At the moment this possibility seems distinctly unlikely – all the evidence indicates that natural antibody belongs to the class of 19S globulin and is identical with the early response 19S antibody first found after active immunization. This has been well studied by SVEHAG[12] in whose hands the physico-chemical characteristics of natural 19S anti-polio antibody were found to be the same as those of the early acquired 19S antibody. Two important points emerge: firstly, both natural and early 19S antibody possess two-stage heat inactivation curves which indicate a two-component system; secondly, the heat stability of immune antibody increases with increasing time after immunization. SVEHAG suggests that the two components in normal serum are 19S antibody and C^1, which, of course, is more heat-labile and has been shown by various workers to be involved in virus neutralization by normal serum. Against this suggestion is the fact that the early stage of heat inactivation could not be restored by addition of C^1. It seems possible that there is another heat-labile component in normal serum, other than C^1, which is required for antibody activity. In the recent studies of HIRSCH and STRAUSS on the opsonic power of normal rabbit serum[13] they found evidence for a heat-labile factor (not C^1) which was necessary for maximum phagocytosis to occur. One of the areas in immunology which is most in need of further work and clarification is that which concerns the antibody activities in the serum of normal, non-immunized animals.

In the scheme which I have outlined to you, the macrophage performs a key function in phagocytosing, digesting and further processing the antigen so that specific information for antibody production can be obtained. There are many infectious diseases of man and animals in which the production of antibody is the decisive factor which leads to recovery or immunity. Can we account for some of the known individual or species differences in susceptibility by variations of this initial phagocytic or digestive role of macrophages. One can see that antibody production might be diminished if any of the steps above involving macrophages were severely restricted for one reason or another.

A very interesting example of a block in immune activity possibly due to macrophages is provided by the recent work using synthetic polypeptide antigens by BENACERRAF et al.[14] among others. They have shown that although 100% of a population of guinea-pigs can respond to the hapten di-nitrophenol when this is attached to a protein, if the same hapten is attached to a synthetic polypeptide such as poly-L-lysine, then only a proportion of the animals are capable of responding. None of the animals produce antibody when the hapten is combined with poly-D-lysine. Moreover, responsiveness was shown by progeny-testing to be a dominant single-gene controlled characteristic. Some of the animals lack the capacity to translate the antigen into information for antibody synthesis although they manifestly do not lack the ability to respond when the same antigenic grouping is provided in the form of the DNP protein complex. Where the defect lies is not yet clear, but this fascinating approach should lend itself to solution.

An obvious reason for lack of phagocytosis of antigenic material would be lack of the initial reactive serum proteins which are usually involved. A possible cause for their deficiency would be a similarity of antigenic structure between host and its parasite. If the necessary anti-parasite antibody were formed, it would be auto-antibody and would tend either to be adsorbed onto host constituents or to eliminate its own further production by means of the controlling mechanisms already discussed. We have suggested that this sharing of antigenic similarities may be an important factor in those infections where the specificity of the host to its parasite is particularly restricted[15]. In other words, one would expect such considerations to be more important in infections such as cholera, smallpox and influenza in man where the host range is extremely limited; in contrast to the infections caused by *Mycobacterium tuberculosis*, Streptococci and Staphylococci which can involve many different animal species with equal severity. When Dr. JENKIN and I put forward this suggestion we chose *S. typhimurium* infections of mice as our example. This is a natural disease to which mice are very susceptible. Injection of as few as 10 organisms may kill 50% of the mouse population, whereas rats and other animals may easily resist 10^7 organisms. We have three bits of evidence for antigenic similarities between mouse and *S. typhimurium*. A rabbit-anti-mouse serum was highly effective in promoting phagocytosis of *S. typhimurium* and this property could be absorbed from the sera with either mouse cells or *S. typhimurium*. Secondly, rats which are resistant to this organism will become quite susceptible if made partially tolerant of mouse tissue by neonatal grafting. In other words when their ability to react immunologically against mouse is diminished, so is their capacity to resist *S. typhimurium*. Finally, mice which have become chronic carriers of this organism following infection, develop a positive Coombs test, their red cells agglutinate when treated with a rabbit antimouse globulin serum indicating the presence on the red cells of reactive globulins[16]. None of

[12] S. E. SVEHAG, J. exp. Med. *119*, 517 (1964).
[13] J. G. HIRSCH and B. STRAUSS, J. Immunol. *92*, 145 (1964).
[14] B. B. LEVINE, A. OJEDA, and B. BENACERRAF, Nature *200*, 544 (1963).
[15] D. ROWLEY and C. R. JENKIN, Nature *193*, 151 (1962).
[16] C. R. JENKIN and D. ROWLEY, Bact. Rev. *27*, 391 (1963).

these data is conclusive and although a good deal of suggestive evidence is now available from other workers this will remain just a nice idea until cross-reacting antigens can be isolated from both host and parasite.

There are other situations in which defective phagocytosis may be the reason for lack of antibody synthesis. It has been shown by DRESSER[17] that centrifugation of γ-globulin, so as to remove aggregated material, greatly diminishes its antigenic powers. That this is presumably due to ineffective phagocytosis is supported by experiments of FREI and BENACERRAF[18] in which they gave rabbits a large dose of albumin intravenously and injected a second rabbit with serum from the first some hours later. The serum, of course, contained albumin, but only that portion of it which was less easily phagocytosed – the recipient rabbits consistently failed to form antibody to the albumin.

My final example concerns the practical and important area of gastro-enteritis in young animals, particularly calves. The disease of calf scours occurs typically in young calves deprived of colostrum and kept under crowded, dirty conditions, an overwhelming enteric infection often results which may be caused by many strains of *E. coli* and much effort has been exerted to try to find characters of these strains which can account for their extreme pathogenicity. The new-born calves have very low amounts of antibody, mostly of the less efficient 7S type. The intestinal epithelium is known to be very well supplied with macrophages at this early age and I suggest that in the presence of copro-antibody these phagocytes can help to maintain the balance of the intestinal flora. Without colostrum, however, the antibody level may be almost limiting and superimposed on this is a high intake of enteric organisms by mouth, many of which will survive the achlorhydric conditions in the stomach of the new-born. These conditions are analogous to absorbing a weak multipotent antiserum with a mixture of organisms; sooner or later one of the strains will have removed all its specific antibody and the phagocytes in the intestine will no longer operate against that strain. I suggest there may be nothing more special about the so-called pathogenic strains of *E. coli* than that these are the ones against which the animal first runs out of antibody. This is a man-made situation in which lack of phagocytosis may be the reason for failure of the immune response.

You will all have realized by this time that I have been deliberately provocative in my observations. But even allowing for my natural bias towards phagocytosis, I hope I may have convinced you that the handling of antigen by macrophages is an important component of the immune response which should not be forgotten.

Zusammenfassung. Die Phagocyten tragen wesentlich zur Immunreaktion bei, indem das Antigen zerstört wird und eine spezifische Reizung der Lymphocyten herbeiführt. Die Makrophagen sind dieser Rolle gut angepasst, da sie in einer ersten Phase der Immunreaktion zunehmen und in einer späteren Phase sowohl zahlenmässig wie funktionell durch einen Überschuss an 7S-Antikörpern herabgesetzt werden.

[17] D. W. DRESSER, Nature *191*, 1169 (1961).
[18] P. C. FREI, B. BENACERRAF, and G. J. THORBECKE, personal communication (1965).

The Kinetics of Phagocytosis*

Ever since the days of METCHNIKOFF and PFEIFFER there have been arguments about the relative importance of cells versus humoral factors in the defence of the body against infection. One might have thought that these had been happily resolved by the demonstration of ALMROTH WRIGHT that for phagocytosis of bacteria to occur efficiently, both cells and serum factors (called opsonins) were necessary. However, the arguments still continue in a slightly altered form and were, I think, revived by the observations of LURIE in 1940[1] that resistance to *M. tuberculosis* in immunized rabbits appeared to be a property of the mononuclear phagocytic cells in the complete absence of any effect of the serum. Since that time the concept of cellular immunity has grown and associated with this have been examples of phagocytosis occurring in the apparent absence of antibody. One of the main difficulties in studying phagocytic mechanisms to delineate the component parts is that, by necessity, one of the components of the reaction is a living cell. And even if the work has utilized cells cultured in vitro, one can never be certain that they do not have important factors derived from their original host adsorbed onto

* This paper represents the *second* of three Warner-Lambert lectures given at Rutgers University in October 1964.

[1] M. B. LURIE, J. exp. Med. *75*, 247 (1942).

their surfaces. This is particularly true with phagocytic cells which have always been used as primary cultures. There is now ample evidence that normal serum proteins cannot be removed from cells by repeated washing in isotonic solutions. To demonstrate that such well-washed cells are capable of phagocytosis in the absence of added serum, is not a satisfactory proof that serum proteins are unnecessary for the event. My point is greatly strengthened by the minute amounts of protein which may be involved, as I will demonstrate. If 10^6 unopsonized (i.e. not treated with antiserum) *S. typhimurium* are injected intraperitoneally into normal mice and the surviving intraperitoneal bacteria are enumerated at intervals, it will be found that little, if any, reduction in numbers occurs within 90 min. If, however, as little as 0.00002 μg of specific 19S antibody is injected together with the 10^6 organisms, then rapid killing will occur. That this is due to phagocytosis and further action by the peritoneal cells, can be deduced by washing out the mouse peritoneum and lightly centrifuging at 500 rpm for 5 min to bring down the cells before counting the viable bacteria in the supernatant fluid. A high percentage of the viable organisms are found to be cell-associated, in contrast to the situation in which no antibody is present.

We can make an interesting calculation from this simple experiment – in each mouse peritoneum there are approximately $5 \cdot 10^6$ macrophages and we injected 10^6 organisms. Assuming a molecular weight of 10^6 for macroglobulins we can say:

$$10^6 \text{ g} = 6 \cdot 10^{23} \text{ molecules}$$

(Avogadro's number)

$$2 \cdot 10^{-5}\,\mu g = \frac{6 \cdot 10^{23} \cdot 2}{10^6 \cdot 10^6 \cdot 10^5} \text{ molecules}$$

$$= 12 \cdot 10^6 \text{ molecules.}$$

This works out at 12 molecules/bacterium or about 2 molecules/macrophage – in either case an incredibly small amount. It is surely never possible to be certain that this number of molecules is not already adsorbed onto the macrophages, in which case addition of more serum would not increase the amount of phagocytosis. You will see from the next paper that it is possible to elute from the washed macrophages of normal or immune mice many thousand times this amount of protein.

This difficulty, due to cell-adsorbed proteins, can only be discounted if, in the technique used to study phagocytosis, there is *no* uptake in the absence of added factors. Such a system obtains when one works with organisms such as the Streptococci, and the results seem clear that without addition of specific anticapsular antibody there is no phagocytosis; in its presence there is rapid uptake followed by intracellular killing. This whole process was studied on a quantitative basis by the delightfully photogenic work of ARMINE T. WILSON who followed this by a combination of time-lapse cinematography and viable counting. He found phagocytosis to be extremely rapid and the subsequent intracellular killing of the bacteria to have a half life of 6–7 min. Over the past six or seven years my colleagues and I have extended these findings of WILSON to other organisms using several techniques but restricting our attention mainly to mouse macrophages. This narrow restriction is for the very good reason that it is only from the mouse that one can conveniently obtain large numbers of peritoneal macrophages without any previous treatment. From other laboratory animals one can only collect sufficient and workable numbers of cells if they have been stimulated by the previous intraperitoneal injection of glycogen, starch or other particulate, phagocytosable material. These have, of course, already engulfed the provoking material and may no longer represent the normal cell.

In our first method we have used the mouse peritoneum itself as our culture chamber[2]; this normally contains several million macrophages, which can be recovered simply by injecting 1.5 ml of tissue culture medium intraperitoneally and sucking this out within a few seconds. The in vivo method consists of injecting a known number of living bacteria intraperitoneally into a group of mice and then recovering the intraperitoneal cells and organisms from individual mice at timed intervals, each interval requiring the sacrifice of at least one mouse. The distribution of bacteria between cells and supernatant is estimated by lightly centrifuging the washout, as described above.

In other techniques we have transferred the macrophages to tissue culture tubes either after phagocytosis has occurred in vivo, so that we could follow the continuing intracellular killing, *or* the whole process has been done under in vitro conditions[3].

The main conclusions we have reached are as follows:

(1) There are many organisms which are not phagocytosed under any conditions unless serum factors have been added. Some organisms are taken up in the absence of added serum and, for the reasons given earlier, one cannot be certain about the requirements for opsonins in these cases.

(2) Once inside the cells all the organisms we have studied are killed at rather similar initial rates, with half-lives around 6–9 min, the one exception being *St. aureus* which gave a half-life of 20 min – a figure in good agreement with the 20–30 min found by MELLY and ROGERS[4] and by MACKANESS[5].

[2] J. L. WHITBY and D. ROWLEY, Brit. J. exp. Path. *40*, 358 (1959).
[3] D. ROWLEY and J. L. WHITBY, Brit. J. exp. Path. *40*, 507 (1959).
[4] M. A. MELLY, J. B. THOMISON, and D. E. ROGERS, J. exp. Med. *112*, 1121 (1960).
[5] G. B. MACKANESS, J. exp. Med. *112*, 35 (1960).

(3) The phagocytic cells are heterogeneous with respect to their bactericidal activity. Conditions in our experiments have always provided a very great excess of macrophages/bacteria so that in the ultimate analysis we have studied single bacterium/single cell interactions. In spite of this the initial rate of killing usually ceased when there were still considerable numbers of macrophages containing single bacteria. I will discuss this heterogeneity more fully in my next lecture.

One of the most noticeable features of work of this kind is that no in vitro system yet designed can approach the intraperitoneal rate of phagocytosis. I believe most people would agree with this. Some experiments of my colleague AUZINS may help us to understand the reasons for the difference in efficiency. She studied the very earliest step in phagocytosis, i.e. adherence of bacteria and macrophages[6]. She mixed opsonized bacteria together with macrophages in suspension and was able to take frequent samples of the free bacteria by passing the suspension through filter paper, which held back the macrophages but not the free, unattached bacteria. She found extremely rapid adherence to occur, but it stopped when 50% of the bacteria were still free. I believe this was due to equilibrium between adsorption and desorption of the bacteria, which can obviously occur in suspension. When the cells are attached to a surface as in the mouse peritoneum the adsorbed bacteria must often be trapped against the endothelial linings and in this way a reversible equilibrium is largely prevented. In other words this is the efficient 'surface phagocytosis' which WOOD has stressed for years[7].

No talk on phagocytosis would be complete without a mention of the pioneer work of BENACERRAF et al. on the kinetics of clearance by the intact reticulo-endothelial system. They have developed quantitative methods for its study which are of great value and have also underlined the importance of antibody for the clearance of bacteria by the reticulo-endothelial system[8]. Bearing in mind the complications due to adsorbed serum factors and allowing for this, we can get a great deal of information from studies of the phagocytic ability of the reticulo-endothelial system in situ. My colleagues HOWARD and WARDLAW tried to define the requirements for phagocytosis using a liver perfusion system in the rat[9]. Briefly they cannulated the portal and inferior vena cava so that the portal circulation of the liver was isolated. Then they ran through suspensions of various bacteria under differing conditions. With *E. coli*, perfused in Ringer-Locke solution, phagocytosis during one pass through the liver was negligible. Addition of fresh rat serum to the perfusion fluid increased the efficiency so that about 40% of the bacteria were removed in one pass. Treatment of the rat serum to remove complement, or absorption of natural antibody in the serum by bacteria, decreased its ability to promote phagocytosis. They concluded that specific natural antibody was by far the most important opsonic factor and that complement, if present, also enhanced uptake. When this work was extended to many other organisms they made the surprising finding that some bacteria did not, by this technique, need any added serum for their removal and indeed there were a few whose uptake was diminished by serum[10]. At the time we had no good explanation of this, but I now wonder if this could be a blocking of antigenic sites on the bacteria by non-complement-fixing antibody of the γ_1-kind which BENACERRAF has demonstrated. It would certainly be interesting to know if excess antibody in vivo could ever act by inhibiting phagocytosis; as we believe it may do with soluble antigens when presented in a complex of the type $Ag/Ab_{(n+1)}$.

Phagocytosis and clearance of foreign material by special cells is a function possessed by most if not all invertebrates, in spite of the fact that these animals do not produce conventional mammalian-type antibody. Since ontogeny and phylogeny often follow related paths there has been much interest in studying the development of reticulo-endothelial system function during embryonic life. In our Department we have followed this in the incubating hen's eggs[11] and in foetal rats[12]. With both systems, the technique has been to follow the rate of clearance of P^{32}-labelled bacteria from the circulation after injection into the allantoic vein of the egg or the anterior orbital vein of the rat foetus. KARTHIGASU and READE, who did this work, were both able to span the last one-third of the gestation period and both studies indicated that phagocytic function in the reticulo-endothelial system develops throughout this period, as does the capacity to form antibody.

All this work indicates that for phagocytosis by mammalian cells to occur efficiently, antibodies or reactive globulins are necessary to sensitize the particulate or antigenic material. HOWARD and WARDLAW's work on liver perfusion had indicated the desirability of complement, and the work of NELSON on immune-adherence[13], which one might think was a necessary preliminary to phagocytosis, had strongly implicated complement in this reaction. Within the past year or so SPIEGELBERG, MIESCHER, and BENACERRAF have turned this possibility into a probability by their work

[6] IEWA AUZINS and D. ROWLEY, Aust. J. exp. Biol. Med. Sci. *41*, 539 (1963).
[7] W. B. WOOD JR., M. R. SMITH, and B. WATSON, J. exp. Med. *84*, 387 (1946).
[8] B. BENACERRAF, M. M. SEBESTYEN, and S. SCHLOSSMAN, J. exp. Med. *110*, 27 (1959).
[9] J. G. HOWARD and A. C. WARDLAW, Immunology *1*, 338 (1958).
[10] A. C. WARDLAW and J. G. HOWARD, Brit. J. exp. Path. *40*, 113 (1959).
[11] K. KARTHIGASU and C. R. JENKIN, Immunology *6*, 255 (1963).
[12] P. C. READE and C. R. JENKIN, Immunology, in press.
[13] R. A. NELSON JR., Science *118*, 733 (1953).

with decomplemented mice[14]. They found that, if the already low levels of complement in mice were further diminished by the injection of heat-aggregated γ-globulin, which is highly anti-complementary, then the rates at which these 'decomplemented' animals cleared bacteria or heterologous red cells were greatly diminished. The original normal rate of clearance could be restored by the injection of fresh normal serum. A possible objection to these experiments could be that the injection of 2.5 mg of aggregated γ-globulin would produce a blockade of the reticulo-endothelial system, due partly to a limitation of opsonic factors against the γ-globulin, and that addition of fresh serum might reverse the effect by the provision of more opsonic factors, thus allowing phagocytosis to proceed. In this case the decrease in complement would be merely coincidental. This explanation now seems unlikely as MIESCHER et al. have produced the same sort of results in vitro where considerations of blockade do not apply. Another finding from the same school which surprised me, is that the various fragments of antibody produced by enzymic hydrolysis will not readily promote phagocytosis. And this in spite of the fact that at high concentrations some of these fragments will produce agglutination and can be shown by other means to fix specifically to the erythrocyte or bacterial surface against which they possess antibody activity. The fragments of hemolytic antibody will not lyse erythrocytes in the presence of complement and this appears to be due to their inability to fix complement[15]. It is reasonable to deduce then that the same fragments will not permit phagocytosis because they cannot fix complement and *therefore because complement is necessary*. This conclusion is also strongly supported by the experiments of NELSON in which he showed that sheep red cells were only phagocytosed efficiently by guinea-pig leucocytes if they were coated with C'1, 4, 2 and 3c components in addition to antibody[16].

In ISLIKER's laboratory in Switzerland we have examined the opsonic activity of antibody fragments I, II and III produced from rabbit anti-*S. adelaide* γ-globulin by papain and cysteine digestion. As a phagocytic test we have used the ability of the mouse peritoneum to phagocytose and kill *S. adelaide* – this is a very sensitive and simple test. Whereas the original γ-globulin was active at 0.1 μg/mouse the fragments showed activity only at 100 μg/mouse. Labelling the fragments with I^{131} enabled us to follow the adsorption of the proteins onto the bacteria at varying concentrations and to show that comparable amounts of fragments or of whole γ-globulin were fixed to the bacteria. In other words the lack of activity of fragments could not be explained solely by a decrease in the amounts fixed to the organism[17]. These results support the conclusions of MIESCHER and BENACERRAF that complement is involved in phagocytosis. Within the last year BENACERRAF et al. have described two electrophoretically distinct 7S γ-globulin antibodies in guinea-pig serum, both having the same antigenic specificity, and named fast and slow or γ_1 and γ_2[18]. Apart from their electrochemical differences these also differ in ability to fix complement, as the γ_1-antibody fails to do this. This γ_1-antibody seems inactive in any of the immunological reactions which normally require complement, such as the Arthus, hemolytic and bactericidal reactions. The only biological methods of detecting the γ_1-antibody are by passive cutaneous anaphylaxis or by competitive inhibition of the complement-dependent reactions which the γ_2-antibody will perform. As far as I know, the definitive experiment using purified γ_1-antibody in phagocytosis has not yet been done, but even as it stands now this work provides strong support for the idea of complement-dependent phagocytosis. And incidentally this provides another possible cause of ineffective immunological responses. Since γ_1 may block the effects of γ_2, an animal responding for some reason with excess γ_1 might be incapable of mounting any of the defence mechanisms requiring complement. ISLIKER recently suggested an experimental approach to me, which has a bearing on this whole question. His idea was to take normal human γ-globulin and to absorb this with *S. adelaide* to remove natural antibody; then to aggregate this by heat, in which state it would fix complement and should also attach itself to the bacterial surface, perhaps stimulating antibody. The question was, would this material promote phagocytosis? We have done this and demonstrated that the anti-complementary power of the aggregated γ-globulin was retained even when attached to the bacteria. Labelling the aggregate with I^{131} enabled us to follow the uptake on the bacterial surface and to find that under our conditions approximately 15% of the offered aggregated γ was adsorbed onto the bacteria. However, when bacteria were treated with 10 μg–2 mg/ml of the aggregate and washed before injection into the peritoneal cavities of mice, there was no evidence of phagocytosis at all. It seemed probable that the aggregated γ remained on the bacteria for the duration of the experiment, since it partially blocked the phagocytosis one would otherwise obtain in mice possessing the specific anti-*S. adelaide* antibody. This was further confirmed by the finding that when organisms and adsorbed aggregated γ were injected into mice im-

[14] H. L. SPIEGELBERG, P. A. MIESCHER, and B. BENACERRAF, J. Immunology *90*, 751 (1963).
[15] K. AMERIAN and E. J. LEIKHIM, Proc. Soc. exp. Biol. Med. *108*, 454 (1961).
[16] R. A. NELSON, Advanc. Immunol. *3*, 146 (1963).
[17] D. ROWLEY, M. THONI, and H. ISLIKER, Nature, in press.
[18] B. BENACERRAF, A. OVARY, K. J. BLOCH, and E. C. FRANKLIN, J. exp. Med. *117*, 937 (1963).

munized against the aggregated γ, there was a rapid phagocytosis and killing.

We must surely conclude from this, that the mere fixation of complement near the bacterial surface is not sufficient to determine phagocytosis; the specificity of antigen/antibody combination is also demanded.

When we recall how few molecules of 19S antibody are necessary on the bacterial surface, we are compelled to abandon the concept that opsonization involves 'coating' the bacteria with a layer of protein and perhaps a modification of total electrical charge distribution. Add to this the requirement for complement which, on a molecular basis, must be of the same order as the number of antibody molecules and we are forced to look for other more specific explanations.

I have given some thought to the possibility that there might be chemical 'spikes' or hot spots on the bacterial surface which must be neutralized by antibody before uptake by phagocytic cells can occur. This has two weaknesses which to my mind make it untenable: firstly, it gives no explanation for the role of complement and secondly, there are many bacteria such as *S. typhimurium* with several distinct antigenic determinants on the surface. In the case of *S. typhimurium* these are classified as I, IV, V and XII by the Kauffmann-White serological scheme. It transpires that single factor antibodies against any one of these antigenic groupings can induce phagocytosis. Thus even if the chemical 'spikes' on antigen IV were covered up by antibody, there would still be those of the other antigens, exposed and repellent. The nature of the antigen against which antibody is directed, seems of minor importance for phagocytosis and the vital step may be the act of antigen/antibody union in the presence of complement and the release of a mediator substance.

It has, in fact, been suggested by ISLIKER and by FISHER that antibody + complement may act, particularly in their cytolytic effects, by the release of a surface active substance such as lysolecithin in close proximity to the cell membrane[19]. FISHER has shown that activation of complement by antigen/antibody reaction is accompanied by lysolecithin formation and there is other evidence correlating lysolecithin levels with those of complement in rabbits. At the moment the evidence for lysolecithin as a mediator of complement action is not good and its involvement in phagocytosis is even more doubtful. It is, nevertheless, an interesting possibility worth further study.

The only work I am aware of which indicates the existence of the sort of mediator I am proposing, is that of BOYDEN[20]. He studied the migration of rabbit polymorphs through a millipore membrane towards antigen/antibody complexes on the other side. He found that in the presence of a heat-labile serum factor (possibly complement) such complexes would exert a strong chemotactic effect on the leucocytes which was shown to be due to release of a heat stable mediator. Macrophages respond to similar chemotactic influences and in the final analysis phagocytosis may be the end result of chemotaxis. The nature of this mediator of Boyden demands further study.

The inability of γ-globulin to promote phagocytosis would be easily understandable if aggregated γ + complement failed to release Boyden's chemotactic mediator. It is interesting that it does not appear to release lysolecithin[21].

Zusammenfassung. Die Notwendigkeit eines Opsonins für die Phagocytose und die Tatsache, dass nur 12 Moleküle γM-Antikörper genügen, um eine Bakterienzelle zu opsonisieren, wird besonders hervorgehoben. Die Ansicht, dass die Opsonisierung ausschliesslich auf einer Belegung von Partikeln mit einer Eiweißschicht beruhe, wird abgelehnt. Es handelt sich um einen spezifischen Mechanismus, wobei zusätzlich zu der Fixierung von Antikörper eine Aktivierung des Komplementes in allernächster Nähe der Bakterienoberfläche für die Phagocytose unerlässlich ist.

[19] H. FISHER, in Ciba Foundation Symposium on Complement (1965).
[20] S. V. BOYDEN, J. exp. Med. *115*, 453 (1962).
[21] J. KELLER, personal communication (1964).

The Carrier State and Cellular Immunity*

As students of immunology we must constantly remind ourselves that there are many phenomena which, although possessing all the specificity of antibody-mediated reactions, nevertheless cannot at the moment be accounted for in terms of classical antibody. I refer, of course, particularly to delayed-type hypersensitivity and to rejection of tissue grafts. Passive transfer of these immunological phenomena from one animal to another can be accomplished by lymphocytes but not by serum. With the advent of the auto-immune diseases, many of which appear to fall into this class of cell-mediated reactions, it has become increasingly imperative to arrive at an understanding of this

* This paper represents the *third* of three Warner-Lambert lectures given at Rutgers University in October 1964.

second kind of immunity. We would like to know whether this really is a whole class of immune reactions due to cellular attributes which are independent of and divorced from classical antibody, or whether they are fundamentally antibody-mediated but the role of antibody being obscured by being bound to cells. In view of the exceedingly small number of molecules of 19S antibody needed for immune cytolysis, it is going to be difficult to separate these two possibilities with certainty in many cases.

Because of our current interest in the subject of infectious diseases, we will confine ourselves to examples in this area. There are many bacterial diseases to which host immunity may develop, in which no protective humoral antibodies can be clearly found. I mention, in particular, tuberculosis and brucellosis of man or animals and *S. typhimurium* or *S. enteritidis* infections of mice. The belief that these fit into the class of 'cell-mediated' immune reactions rests on evidence similar to that of LURIE[1] in his demonstration that immunity to *M. tuberculosis* in rabbits was a property of the mononuclear cells and had nothing to do with serum factors. This was further strengthened by the fact that immunity to each of these diseases could be produced by vaccines of living attenuated organisms but not by killed bacteria, in direct contrast to most of the recognizably antibody-mediated examples of bacterial immunity.

This 'cellular immunity' with which we will now concern ourselves is highly specific. JENKIN and I chose *S. typhimurium* infections of mice, some years ago, as our model of this condition. This, as you know, is a natural epidemic disease of mice which can be initiated experimentally by the injection of as few as 10 organisms. Good specific protection may be obtained with a living attenuated vaccine and, as shown by HOBSON[2], this results in a stable 'carrier state' in which parasite and host live in balanced harmony for long periods. Moreover, several workers have claimed that immunity to this infection, whether of the natural interspecies kind or acquired within the one host species, may be correlated with the ability of the macrophages to suppress intracellular growth of the organism. USHIBA et al.[3], working with the closely related *S. enteritidis* infection of mice, have been unable to transfer immunity with serum, but the peritoneal macrophages from immunized mice appear able to confer resistance to normal recipients[4]. With all this background information one might fairly claim that this disease comes into the class of cell-mediated phenomena and indeed this claim has been made often before by others. I propose now to try to destroy this claim, at least in so far as it implies new cell properties due to reactions other than antibody-involved ones.

In our studies we have used an avirulent vaccine straine named M_{206} and a virulent strain C5 for challenge. Mice given 10^6 M_{206} by any route, quickly developed resistance to challenge by many lethal doses of C5 and this lasted for a long time, during which small numbers of M_{206} organisms could be recovered from the spleen of most animals. The immunity is accompanied by a concurrent change in the ability of the RES to remove the virulent C5. This indicates our basic biological system which we will try to disentangle.

In the first instance we have attempted passive transfer experiments with serum from immune mice. We must remember that mouse globulins have very short half-lives of the order of two days for 19S material and that this infection lasts from 6–21 days before death or survival is finally determined. For these reasons we have given repeated injections of 0.2 ml of 14-day immune serum to a group of 40 mice before and during infection with ~ 200 LD_{50} of C5. Significant protection can be achieved by this means; a protection which is of similar degree to that induced actively by the living vaccine. This by itself, would have been strong evidence for humoral immunity, but we did more experiments which upset this easy solution. When peritoneal macrophages obtained from immunized mice were examined by in vitro methods for their ability to phagocytose and kill an inoculum of *S. typhimurium*, we obtained different answers depending on the time after immunization at which the macrophages were harvested. Fourteen days after immunization the cells behaved as though they were immune in the absence of serum, whereas 4 months later the outcome was determined solely by the presence or absence of immune serum from the phagocytic cell culture[5].

A clue which helped us to explain the puzzle came from studying antibody production by mice after vaccination with living M_{206}. The biological-antibody activity of the sera was determined by an in vivo clearance test, in which the maximum dilution of serum was found which produced a standard increase in the rate of clearance of P^{32}-labelled *S. typhimurium* C5 from the circulation of mice. Electrophoretic and protein separations were done on the serum samples at various times. At 14 days most of the biological activity could be recovered in a macroglobulin fraction which was sensitive to $0.1M$ mercapto-ethanol and had a sedimentation constant around 19S. At two months all the biological activity occurred in normal 7S γ-globulin which was insensitive to mercapto-ethanol. By plotting the total and ME insensitive antibody at varying times after immunization one can

[1] M. B. LURIE, J. exp. Med. *75*, 247 (1942).
[2] D. HOBSON, J. Hyg., Camb. *55*, 334 (1957).
[3] D. USHIBA, Tohoku J. exp. Med. *76*, 133 (1962).
[4] I. SATO and S. MITSUHASHI, Proc. Jap. Acad. *39*, 697 (1963).
[5] C. R. JENKIN, D. ROWLEY, and I. AUZINS, Aust. J. exp. Biol. Med. Sci. *42*, 215 (1964).

see that a phase of maximum 19S antibody yield corresponds with the time of apparent 'cellular' immunity[6].

What is the nature of the immunity seen in 14-day macrophages? Cells taken at this time were thoroughly washed with tissue culture medium and then transferred in an amount equal to the yield from two donor mice to the peritoneum of normal mice. The ability of these recipients to phagocytose and kill i.p. injected *S. typhimurium* is equal to that found in immune mice – in complete agreement with Japanese workers USHIBA and SAITO. Although small quantities of immune mouse serum will produce the same effect, normal macrophages fail to do so. To our surpise, freezing and thawing the washed immune cells eight times did not remove the ability to transfer immune clearance, although no viable cells remained. Centrifugation brought down the activity with the cell debris, leaving an inactive supernatant. We have tried various procedures for eluting the activity away from this cell debris, the most successful being incubation at 37° for 1 h with 2*M* urea. From a batch of 300 14-day immune mice approximately 12 mg of protein were eluted by urea, and 80 µg/animal of this impure material were sufficient to confer immune type of clearance to normal mice. Treatment with 0.1*M* ME completely abolished the activity and centrifugation of one eluted batch of protein in a sucrose density gradient showed that the sedimentation characteristics were compatible with the material being macroglobulin[7]. Comparable amounts of similar but inactive material could be recovered from the macrophages of normal mice. This must be able to exchange with active material, however, since treatment of normal macrophages with active eluted macroglobulin for 1 h, followed by prolonged washing, endows the normal macrophages with the ability to promote rapid intraperitoneal clearance. In this one, deliberately chosen, supposedly typical case of 'cellular immunity' to an infectious agent, the immune state is clearly due to cell-bound antibody, mostly of the 19S variety. It is likely, as EISEN and KARUSH have emphasized recently, that all antibodies have characteristic distributions between the cell-bound and the free unadsorbed state[8]. In those immunological situations where the effect seems cell-mediated, this equilibrium may lie mainly on the cell-bound side and, if coupled with this, only small amounts of antibody are produced; it seems quite possible that virtually the whole of it might be attached to cells. Careful scrutiny of other examples of cell-mediated phenomena, such as delayed-hypersensitivity and graft rejection, might be rewarding. And indeed evidence for cell-bound or cytophylic antibody was provided by BOYDEN and SORKIN long before our own work[9]. What these authors did in effect was to show that humoral antibody against all sorts of antigens is heterogeneous, in that a small proportion of it has affinity for many kinds of normal cells and this portion is often different from the precipitating antibody. In earlier studies they worked with serum from immunized animals which could only contain the excess of cytophylic antibody after all the host's own cells had been saturated. This cytophylic antibody appears to be entirely 7S although the significance of this may be doubtful since it seems to me from their data that probably less than 1% of the cell-bound protein is specific antibody. Nevertheless BOYDEN and SORKIN have realized the full significance of their findings and have suggested that this cytophylic antibody may play much the same role in immunological reactions, such as hypersensitivity and graft rejection, as I have postulated.

Now I would like to return to our example of mouse typhoid. I have explained one of the difficulties which faced us earlier, but what about the fact that one cannot produce effective immunity with killed vaccines? This is a less troublesome one, and in all three infections which I have cited there is recent evidence for important labile antigens. RIBI has disrupted *M. tuberculosis*, under oil, taking great care to avoid oxidation at a liquid-air interface, and the cell walls which he obtained were as effective as living BCG in immunizing mice against later challenge[10]. Similarly SMITH, KEPPIE et al. have recently reported the isolation of good immunizing fractions from Brucella cell walls when the organisms had been grown and disrupted in controlled ways[11]. In our laboratory we have been convinced for a long time that the heat-stable O-antigens of *S. typhimurium* were not important protective immunizing agents. With the demonstration by STAUB that the heat-labile antigen V of Salmonella probably involves O-acetyl galactose as a determinant, we have looked for methods of extraction which would preserve this heat and alkali labile O-acetyl structure. In this way we have successfully obtained fractions which are effective in immunizing mice against many lethal doses of *S. typhimurium*; at the moment these are very impure and not at all characterized chemically[12].

In the last paper I touched on the question of macrophage heterogeneity with respect to phagocytosis and killing. I would like to elaborate this important point a little. When mice are injected with virulent C5 the organisms mostly disappear from the

[6] K. J. TURNER, C. R. JENKIN, and D. ROWLEY, Aust. J. exp. Biol. Med. Sci. *42*, 229 (1964).

[7] D. ROWLEY, K. J. TURNER, and C. R. JENKIN, Aust. J. exp. Biol. Med. Sci. *42*, 237 (1964).

[8] H. N. EISEN and F. KARUSH, Nature *202*, 677 (1964).

[9] S. V. BOYDEN and E. SORKIN, Immunology *3*, 272 (1960).

[10] E. RIBI, C. LARSON, W. WIGHT, R. LIST, and G. GOODE, J. Bact., in press (1966).

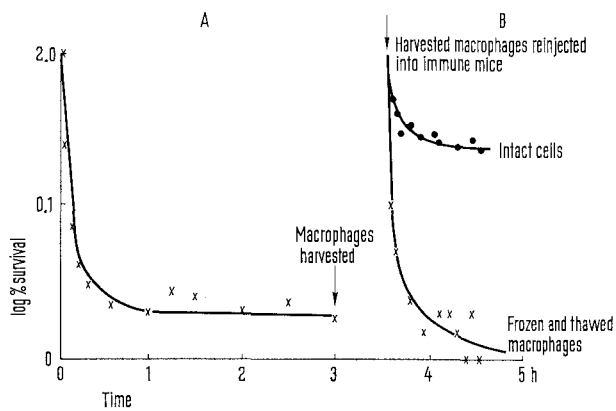
[11] H. SMITH, J. KEPPIE, J. H. PEARCE, and K. WITT, Brit. J. exp. Path. *43*, 538 (1962).

[12] C. R. JENKIN and D. ROWLEY, Aust. J. exp. Biol. Med. Sci. *43*, 65 (1965).

circulation for a day or two, but a few days before the animal dies they reappear in the circulation in very large numbers and, of course, at this time the animal begins to look very sick. If, at this time of bacteraemia, one injects into such mice genetically or isotopically labelled organisms of the same strain, one can find that these are cleared by the RES at a rate much faster than normal. The animal has developed at the peak of its illness (7–10 days) all the requirements for rapid RES function (both antibody and RE cells, since the spleen has increased in size). And yet it is clear from the experiments of many workers that it is from the RES that the bacteraemic organisms reappear. The same organs or tissues are simultaneously seeding and removing large numbers of the same microbes. Since a more or less steady state of bacteraemia exists, one can easily calculate that several million organisms are being released each hour and an equivalent number of the same organisms removed by the RES. This seems to indicate that in the RES effective and ineffective cells may co-exist side by side. We have looked for other evidence to support this rather radical thought, and have found it by following the i.p. killing of genetically marked strains.

A group of 60 mice was infected with 10^6 M_{206} (avirulent); three days later, at a time when they were just acquiring immunity, 40 of these mice were given a second i.p. injection of the virulent Sr C5. Pairs of mice were sacrificed at intervals, their peritoneal cavities washed out, and an aliquot of this plated onto nutrient agar and onto streptomycin agar to differentiate the bacteria of the first and second challenge. Throughout the whole of the experiment an average of 5000 M_{206} was recovered from each peritoneum, yet in spite of this a rapid removal and killing of C5 took place. After following the survival curve for several hours it was apparent that there was little further killing of C5. At this time the remaining 16 mice were killed, and the macrophages harvested and pooled. After washing, the cell suspension was divided into two halves, one of which was frozen and thawed rapidly. Bacterial counts were made at this time one both halves of the suspension and then 0.1 ml amounts of the two suspensions were injected into the remaining 20 mice which had originally received M_{206}. Once again washouts were made at intervals and the surviving bacteria enumerated on both nutrient and streptomycin agar. It is apparent (Figure) that the streptomycin-resistant C5 which persist following the very rapid killing phase, do so because they are within cells which are unable to kill them even under the best opsonic conditions, thus revealing the heterogenetiy of the macrophage population. You may recall that it has been noted that the enzymic and granule content of macrophages could vary considerably according to their recent phagocytic experience. It may be that this is sufficient to explain the variation from cell to cell in bactericidal potential. In order to appreciate fully the significance of this cell component and to avoid the misconception that phagocytosis is necessarily followed by death of the intracellular bacteria, we have tried to put bacteria into cells without first having been in contact with antibody and complement. This, of course, is difficult to achieve since macrophages will not voluntarily take up bacteria under such conditions. My colleague JENKIN has some interesting data in this connection[13]. He persuaded macrophages to engulf *S. typhimurium* by the ingenious device of forming a phage-bacterium complex with a phage to which the organism had been made resistant. Then by adding antibody against the phage, phagocytosis occurred and the bacteria were carried into the macrophages by a 'piggy-back' effect. Under these conditions the bacteria were not affected in viability by their intracellular residence. It seems that the bacteria themselves need contact with specific serum factors before phagocytosis, otherwise intracellular killing will not occur. What better candidates for these serum factors than antibody and complement!

OSAWA and MUSCHEL have shown that lysozyme and the complement bactericidal system may act in synergistic fashion and that gram negative bacteria grown in restricted ways, such as at high temperatures, may become very much more sensitive to this system[14]. This they associate with a decreased synthesis of cell-wall material so that the organisms become phenotypically rough. Several years ago it was shown that



The heterogeneity of the macrophage population with respect to their ability to kill a streptomycin resistant mutant of the virulent strain C5. A, Intraperitoneal survival of the virulent strain of *S. typhimurium* C5 within the peritoneal cavity of immune mice. B, The survival of *S. typhimurium* recovered from the peritoneal cavity of immune mice as indicated in A and transferred, with or without freezing, to the peritoneal cavity of immune recipients. Bacteria released from cells by freezing were more effectively inactivated than those contained in the intact cells in which they were presumably transferred.

[13] C. R. JENKIN, Brit. J. exp. Path. *44*, 47 (1962).
[14] E. OSAWA and L. H. MUSCHEL, J. Immunol. *84*, 203 (1960).

rough mutants derived from smooth gram negative organisms were always more sensitive to the complement bactericidal reaction than their smooth parents[15]. My colleague ALI found that growth of many bacteria in the presence of phloridzin for a short time does the same thing[16]. We can conclude that altering the composition of the bacterial cell wall may greatly change the complement susceptibility.

Now I will try to offer a short synthesis of the events leading to the killing of bacteria by phagocytic cells. There are in this respect two main classes of bacteria: those which are rapidly and inevitably killed following phagocytosis, and here the only limiting factor may be the antibody and complement necessary for uptake (examples of this type are many: Streptococci, Meningococci, Staphylococci etc.), and in the second class, whilst antibody and complement are still necessary for phagocytosis to occur, the subsequent intracellular killing requires the concurrent action of the hydrolytic enzymes in the phagocyte acting at an acid pH; this presumably strips off some of the cell wall material. But in addition to this there is a requirement for something acquired from the serum before entry, and this is possibly the antibody and complement which are in any case vital for phagocytosis. These can then act inside the macrophage in a manner exactly analogous to the well known but more restricted serum bactericidal effect of antibody and complement. Unfortunately for the host there are some macrophages which are defective in this process, possibly due to a shortage of enzymes. But the immune response is so beautifully balanced that the initial contact of the bacterial antigen with antibody and complement at the macrophage surface initiates a rapid multiplication of cells, leading to the provision of a population of macrophages whose ability to kill bacteria has been shifted towards the 100% effective level. This may be due to the increase in enzyme content of these new cells. I believe this shift in cell population accounts for a considerable component of non-specific immunity, in which one can produce a low level of resistance to a variety of unrelated parasites by exposing the host to phagocytosable colloidal materials, like lipopolysaccharides or certain bacteria such as BCG, Brucellae, and *Listeria monocytogenes*.

I do not wish to overemphasize either one of these parameters of host resistance to bacteria. I would only stress that the antibody provides the only specificity in the process, but that cells with their enzymic armoury are needed to complete the destruction of the bacteria. It is well to remember that modifications to one or other of these equally vital, interdependent parts may completely alter the resultant whole process[17].

Zusammenfassung. Es werden Beispiele von Immunreaktionen untersucht, in welchen die Erscheinungen nicht eindeutig auf zirkulierende Antikörper zurückgeführt werden können (Spätreaktionstyp). Eine «zelluläre Immunität» wurde in diesen Fällen, sowie in der Tuberkulose, Brucellose und Salmonellose in Betracht gezogen. Im Falle von *S. typhimurium*-Infektionen der Maus wird die durch abgeschwächte Vaccine herbeigeführte Immunität auf spezifische Antikörper zurückgeführt, die unter gewissen Umständen zellgebunden sein können. Es wird vorgeschlagen, dass auch in anderen Fällen die «zelluläre Immunität» schlussendlich auf zellgebundene Antikörper zurückzuführen ist, obwohl die Immunität nicht notwendigerweise an die Anwesenheit einer Trägerzelle gebunden ist. Möglicherweise ist die Spezifität der Immunreaktion in letzter Analyse immer auf Antikörper zurückzuführen.

[15] D. ROWLEY, Brit. J. exp. Path. *35*, 258 (1954).
[16] D. ROWLEY and A. C. WARDLAW, J. gen. Microbiol. *18*, 529 (1958).
[17] Much of the work from the author's laboratory has been supported by a grant from the U.S.P.H.S. No. AI 03226-05. The lectures were written whilst working at the Institute of Biochemistry in Lausanne (Switzerland) and I am most grateful to the Swiss National Science Foundation for support and to Professor H. ISLIKER for his generous hospitality.