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The specificity of cellular immunity*

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INTRODUCTION

In diseases such as tuberculosis, pasteurellosis, pertussis, brucellosis, salmonellosis, listerosis and many viral infections, where the infecting organism occupies a predominantly intracellular position, protective antibody, even if it is produced, seems to be denied effective immunological contact with the pathogen (Lurie, 1936, 1950; Osebold & Sawyer, 1957; Kadull, Reames, Coriell & Foshay, 1950). However, as Mackaness (1962) has pointed out, unless circulating antibody can be excluded as a factor in acquired immunity, difficulties arise in assessing the actual significance of non-humoral factors, even though these may be playing the major role. One indirect piece of evidence that a significant degree of immunity can be achieved in the virtual or complete absence of antibody is provided by the response of agammaglobulinaemic patients to diseases of the type under discussion (Bruton, 1952). For the purpose of the present discussion it is accepted that a degree of cellular immunity is probably a feature of all immune responses and that it is independent in origin of the plasma cell system responsible for antibody production that may or may not function concurrently with it (Gray, 1964).

In the case of tuberculosis, cellular mechanisms certainly appear to play a major role (Mackaness, 1954) and while there have been several reports of humoral tuberculostatic factors (Dubos, 1954; Seibert *et al.* 1956; Zitrin & Wasz-Hockert, 1957) there is little convincing evidence (Raffel, 1961) that these factors take the form of specific protective antibodies, or even that they are necessary for opsonization, since phagocytosis occurs quickly and completely in the non-immune animal (Gray, 1959).

The degree of specificity underlying cellular immunity has recently been under discussion (Rowley, 1962; Mackaness, 1962). While it is evident that cellular immunity normally follows a specific antigenic stimulus, a phenomenon of current interest to immunologists is the discovery that an increase in resistance to the type of infection under consideration may also arise following several kinds of non-specific stimulus involving unrelated microbial antigens as well as substances presumably unrelated to microbial antigens in any way. These include:

(*a*) Unrelated intracellular infections (Lurie, 1939; Dubos, 1954; Edsall, 1955; Parry, 1956; Rowley, 1956; Smith, 1956; Mackaness, 1962).

(*b*) Bacterial lipopolysaccharides (endotoxins), particularly those derived from Gram-negative bacteria (Westphal, 1957; Boehme & Dubos, 1958; Rosen, 1961).

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(*c*) Certain simple lipids, such as the triglycerides, tri-olein or tri-caprin (Stuart *et al.* 1960; Cooper & Stuart, 1962; Cooper & West, 1962; Stuart & Cooper, 1962).

(*d*) Certain surface-active detergents represented by polyoxyethylene ethers (Cornforth, Hart, Rees & Stock, 1951; Hart, Long & Rees, 1952; Solotorovsky & Gregory, 1952; Mackaness, 1954; Niffeneger & Youmans, 1960).

It is pertinent to question whether these stimuli were all acting on the same cellular mechanism to induce the same phenomenon, or whether increased cellular resistance may be the product of a number of different factors and systems. Taking tuberculosis as a model, since it is the subject of the present paper, the important change occurring under both specific and non-specific stimulation appears to be in the ability of macrophages to kill ingested bacilli, since phagocytosis in itself is never a problem in this disease. This implies that macrophages must undergo a period of training, differentiation, induction or selection, culminating in the emergence of a large population of actively bacteriolytic cells. Incidentally, and this is of importance in assessing reports on the duration of non-specific immunity discussed below, it must be remembered that the period of active bacteriolysis is confined to the first week or two of the immune phase and thereafter is replaced by a steady state in which multiplication and bacteriolysis maintain an equilibrium (Affleck & Gray, 1957; Cheers & Gray 1964).

As has just been implied, the literature suggests the existence of certain apparent points of difference between specific and non-specific cellular immunity requiring further study. Boehme & Dubos (1958), for example, pointed out that the protective effect of endotoxin against *Mycobacterium fortuitum* lasts longer than the increased reticulo-endothelial activity promoted by the endotoxin, and Howard, Rowley & Wardlaw (1958) reported that the non-specific activity was of shorter duration than specific immunity, usually lasting only 7–10 days. Comparing our own observations on specifically induced antituberculous immunity with those of Cornforth and others, it also seemed that the non-specific stimulation may induce increased resistance a good deal more rapidly.

Of immediate practical importance is that, if an effective and durable cellular immunity could be induced non-specifically, it would have a profound effect on current immunoprophylaxis against those diseases for which living vaccines, with their attendant risks, are commonly employed.

The experiments reported here were proposed by Hart in 1960 (personal communication). It had been shown previously that the detergent Triton* enhanced the resistance of guinea-pigs to tuberculosis and apparently suppressed tuberculin allergy in these animals (Hart *et al.* 1952). At the time, Hart & Rees (1960) had also just completed experiments showing that Macrocyclon, a surface-active agent similar in many ways to Triton, suppressed the acute, but not the chronic stage of the disease in intravenously infected mice. Hart suggested that these results might profitably be compared with those obtained from intranasally infected mice, in which changes in the disease pattern with the onset of the immune phase are customarily followed by means of culturable lung counts and footpad tuberculin

* Triton, WR 1339 (Rohm and Hass Co., Philadelphia).

tests (Gray & Jennings, 1955). In this way it was likely that some evidence might be obtained of the possible interference of Triton with the process of antituberculous immunity.

MATERIALS AND METHODS

Experimental mice. Two strains of mice were used: (*a*) C57 Black mice originally obtained from the Roscoe Jackson Memorial Laboratories in 1947, and since randomly mated; and (*b*) MUA (Melbourne University albino) mice, randomly mated for 15 years. The results were similar and apparently independent of strain differences in immunity (Gray, Graham Smith & Noble, 1960). They were fed on Barastoc pellets* supplemented twice weekly with green lucerne. Water, to which 250 mg./l. of chloramphenicol was added to control intercurrent infection with *Corynebacterium murium*, was available *ad lib.*

Infection of mice and assessment of disease. Mycobacterium tuberculosis, strain H37Rv, freeze-dried for storage after animal passage, was grown in dispersed cultures of Tween-albumin medium. Serial dilutions of coarse-filtered, 10-day cultures consisting predominantly of single cells were prepared as previously described (Gray & Mattinson, 1952). This 1952 paper also described in detail the culturable counting technique used and the precautions associated with the intranasal infection of anaesthetized mice. The footpad tuberculin test, using 1/25 old tuberculin in saline, was described by Gray & Jennings (1955).

Triton WR 1339. Distilled water was added to 12·5 g. of Triton in a beaker to make up 50 g. and the mixture stirred during gentle heating. This produced a cloudy mixture which cleared on cooling to give a 25% solution. An equivalent volume of normal saline was added to provide a stable solution of 12·5% concentration. This was autoclaved at 10 lb. for 10 min in flasks which were shaken during cooling to maintain solution of the detergent when cold.

Triton was administered by intraperitoneal injection as either a 10 or 12·5% solution, using a tuberculin syringe fitted with a 26-gauge needle. In a series of tests to confirm the findings of earlier workers, which need not be recorded here, the antibacterial activity of Triton *in vivo* became evident about 5–7 days after a dose of 25 mg. and was adequately maintained by repeating this dose every 2 or 3 weeks. Toxic effects characterized by muscular spasms and some deaths first occurred after the use of 40 mg. twice weekly, by the time the fourth dose was given.

RESULTS

In vitro *antimycobacterial activity of Triton*

The reported absence of any *in vitro* antituberculous activity by Triton was confirmed for the H37 Rv strain used in these laboratories, by exposing heavily inoculated Loewenstein slope cultures to assay disks impregnated with the detergent solution in concentrations ranging from 1 to 20%. After 4 weeks' incubation no inhibition had occurred in the vicinity of the highest Triton levels to which the culture was exposed.

* Barastoc Grower's Pellets—a growing ration for chickens—W. S. Kimpton and Sons, Melbourne.

The influence of Triton on footpad conversion rate

During the course of these experiments, Triton was administered at several different dose levels (25–80 mg./week) to mice infected intranasally with small, medium or large doses of tubercle bacilli, commencing 5 weeks before, during, or shortly after infection and often continuing through the course of the disease. In every experiment records were made of the allergic state of the mice, using bi-weekly footpad tests immediately before selecting those to be killed. Whereas these mouse experiments failed to show any reduction in the degree of tuberculin allergy similar to that reported by Hart and his colleagues (1952) in treated guinea-pigs, in certain combinations of infecting dose and Triton administration there was a significant delay observed in the appearance of measurable allergy.

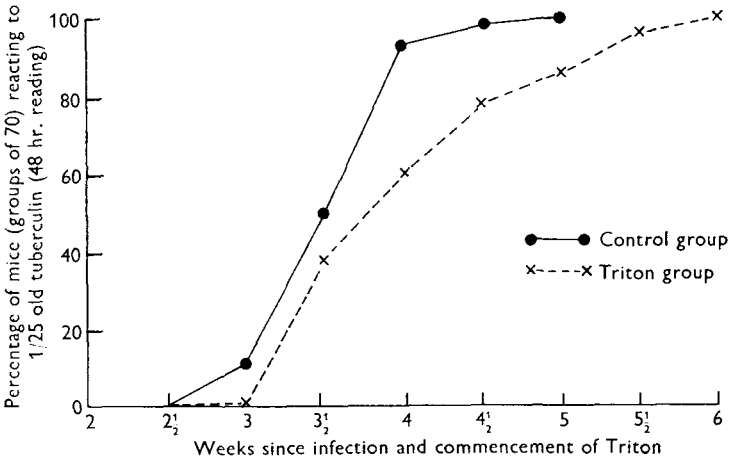

Fig. 1. The influence of Triton WR 1339 on footpad conversion rates in tuberculous mice receiving a small infecting dose. Infecting dose: 150 culturable units of *M. tuberculosis* (H 37 Rv). Triton dose: 25 mg. every 3 weeks, commencing at infection.

Where the infecting dose was small (150 culturable units) and commenced concurrently with the Triton treatment (25 mg. at 3-week intervals) a significant reduction occurred in the conversion rate. A typical result is presented in Fig. 1, where the conversion of groups of seventy mice with and without Triton are compared. However, when the infecting dose was increased above 1000 units the apparent difference in conversion rate became unpredictable, while a very large infecting dose ($2{\cdot}25 \times 10^6$ units) gave a uniform and more rapid conversion in both treated and control mice.

These findings, assessed in relation to earlier work on the influence of infecting dose size on conversion (Gray & Jennings, 1955) strongly suggest that the observed delay in this instance was due to the retarded progress of the disease in Triton-treated mice when the initial dose was small. There was no real evidence that even large and prolonged Triton dosing suppressed the severity of the tuberculin reaction in individual mice, as judged by measuring the thickness of the footpad swelling. It seems possible in the light of these findings that the reduction in

guinea-pig reactions comparable with that produced by cortisone which was reported by Hart and others (1952) might well be masked in rats and mice by their higher allergic threshold (Gray & Jennings, 1955; Gray, Noble & O'Hara, 1961).

The effect of Triton on the progress of pulmonary tuberculosis

We now have a very clear picture of the normal course of murine pulmonary tuberculosis induced by small intranasal infecting doses (Gray & Affleck, 1958; Gray, 1959; Gray *et al.* 1960). An initial lag of about 5 days gives way to rapid multiplication within macrophages lasting from $3\frac{1}{2}$ to 6 weeks, when it is followed by a dramatic reduction of the lung population by well over 90%. This bacteriolytic

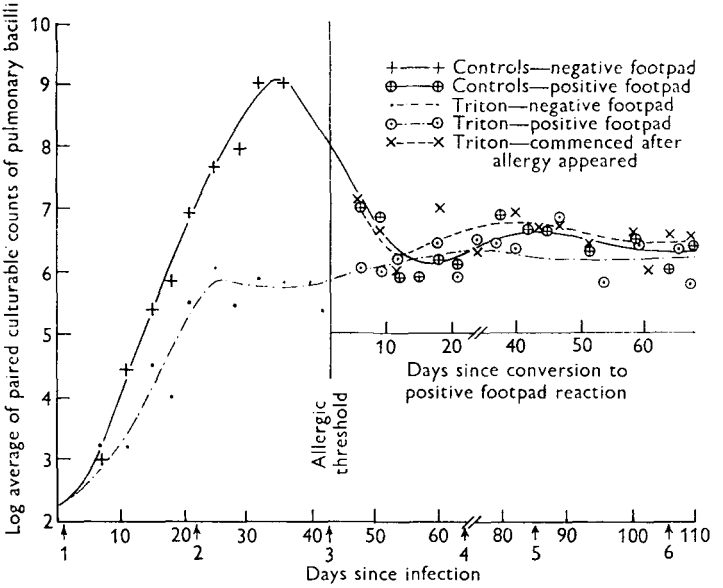

Fig. 2. Effect of Triton on pre-allergic and immune phases of murine pulmonary tuberculosis induced with a small infecting dose. Infecting dose: 150 culturable units. Triton dose: 25 mg. every 3 weeks ($\uparrow_1$, $\uparrow_2$, etc.).

phase, in turn, is quickly replaced by a steady state of usually long, but unpredictable duration (the immune phase), which is normally paralleled by tuberculin allergy. This typical picture is illustrated by the control group, Fig. 2, in which the pre-allergic peak of log 9 was followed by a steady state between logs 6 and 7.

(*a*) *Small infecting dose, with Triton treatment started in pre-allergic or allergic phase*

This trial is typical of several tests on both C 57 and MUA mice, which will not be recorded because of the essential agreement obtained in each case. The conditions included infection with 150 units, and while one treated group of seventy mice received 25 mg. of Triton the following day and again after 3 and 6 weeks, the other group, of similar size, received no Triton until they had converted to the allergic state. The control group received no Triton at any stage.

Two mice from the appropriate groups were killed every 3–4 days immediately

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the results of the concurrent tuberculin tests were known, and the bacilli in the lungs of these were counted, together with those of any mice which died. For convenience of presentation and interpretation, only tuberculin-negative mice were killed in each group as long as they lasted. Meanwhile mice showing positive footpad tests were identified and held. When they were killed it was in order of conversion, so that the times on the graph to the right of the line marked 'Allergic threshold' represent the number of days that had elapsed between conversion and sacrifice in each case.

(1) It will be observed from the results recorded in Fig. 2 that the control mice followed the anticipated pattern, reaching the steady state of the immune phase with a lung population between 10^6 and 10^7 bacilli and remaining there with minor fluctuations throughout the experiment.

(2) The group receiving Triton concurrently with infection not only failed to show a pre-allergic peak, but also entered the steady state on the average 3 weeks after infection, i.e. at least 2 weeks before the controls did so. Although their culturable counts during these 2 weeks were at least as low as those reached later by the immune controls it is noteworthy that these mice were still negative reactors to tuberculin.

(3) The effect of Triton if any, equalled but did not exceed that of the normal immune phase, except in appearing earlier.

(4) Triton administered to mice already in the immune phase did not enhance the existing immunity.

(5) These findings are compatible with an hypothesis that the action of Triton is to accelerate the onset of cellular immunity.

(*b*) *Large infecting dose, preceded or accompanied by Triton*

The large infecting dose was used to determine whether the delay in conversion reported above was due merely to the small number of bacilli lysed at the beginning of the immune phase. It was also of interest to determine the degree of protection afforded against death when a combination of high infecting and low Triton dose was given and whether it would influence the relative number of organisms surviving to the steady state in the treated animals. In this experiment, the infecting dose of H 37 Rv selected was $2{\cdot}25 \times 10^6$ units accompanied by a single, 25 mg. dose of Triton. The results which are set out in Fig. 3 have been interpreted as follows:

(1) The single dose of Triton administered protected a high proportion of the heavily infected mice against the chances of pre-allergic death (4/40 deaths compared with 13/40 in the control group). It must therefore be concluded that the protective effects of the single dose persisted in most mice through the pre-allergic phase. No observations have yet been made to ascertain whether higher or repeated dosing with Triton would have increased the resistance at this infecting dose level.

(2) The Triton-protected mice, although they tended to survive the pre-allergic stage, were less well able to restrain pre-allergic multiplication than mice receiving a smaller infecting dose (see Triton group, Fig. 2).

(3) Allowing for a tendency for heavy-handed procedures to dampen minor differences, this experiment requires no significant modification of the interpretations based on the smaller infecting dose (Fig. 2). It will be noted that, although more treated than control mice entered the immune phase, there was again no significant difference between the steady state levels reached by the two groups.

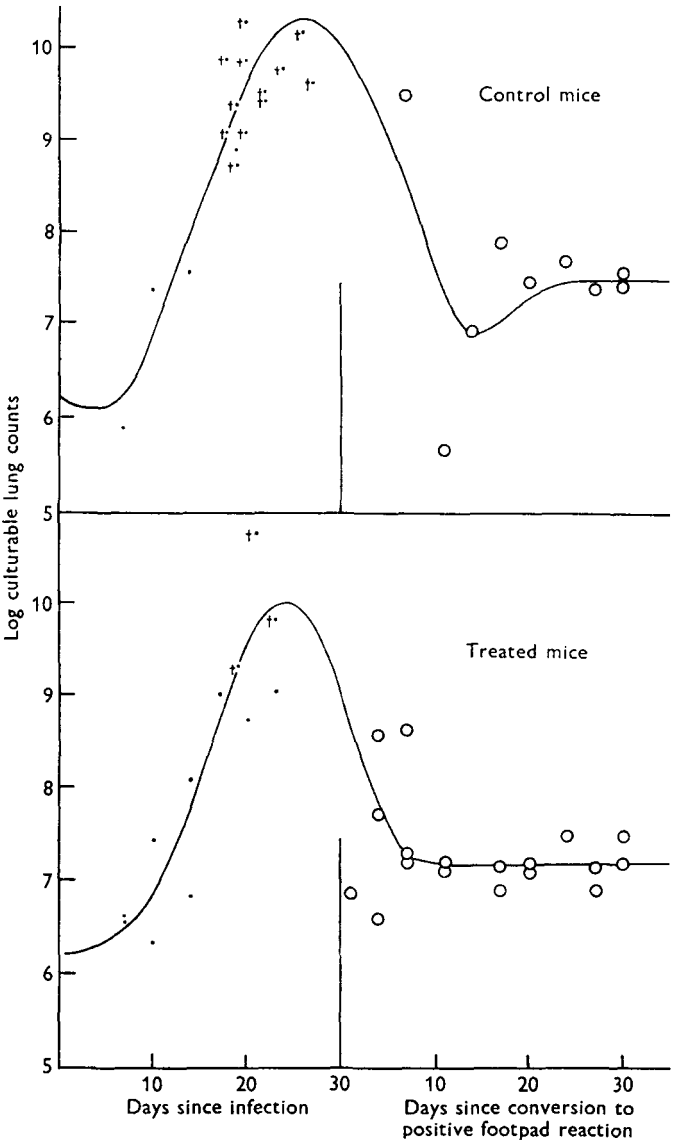

Fig. 3. Influence of heavy infecting dose on protective action of Triton. Infecting dose: $2{\cdot}25 \times 10^6$ culturable units. Triton dose: Single dose of 25 mg. coinciding with infection. † = Died. Pre-allergic deaths = 13/40 controls; 4/40 treated mice.

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DISCUSSION

The most important obstacle to a clearer understanding of the nature of cellular immunity does not appear to be concerned with opsonization and ingestion of bacteria, but rather with the changes occurring in the intracellular activities of macrophage cells which convert tolerance into bacteriolysis. This is perhaps best illustrated by chronic infections such as tuberculosis, in which normal macrophages rapidly ingest tubercle bacilli and allow them to multiply freely for several weeks before any detectable bactericidal properties are acquired. It is not clear whether this represents a period of training for existing cells, or one of replacement by selected or educated immunologically competent cells. Moreover, how widely and how completely this acquired competence embraces enhanced resistance against other intracellular pathogens is still under discussion (Crowle, 1963).

A puzzling feature is the variation in time needed to reach a state of competence under different conditions. As we have seen, in mice infected with tubercle bacilli or immunized with BCG, it may take from 3 to 6 weeks to develop bactericidal properties, but lipopolysaccharide or Triton can apparently produce the same antituberculous efficiency within a few days. Moreover, specifically acquired competence against *Listeria monocytogenes* and *Salmonella typhi-murium*, which are stated to afford cross-protection for tuberculosis, can be developed in a few days. The question of whether training or replacement of macrophages occurs is perhaps of less immediate importance than the probability that these competent cells can be induced to appear in the body at varying rates and be maintained for varying periods by a group of unrelated, specific and non-specific stimuli. These appear to have little or nothing in common and some of them must be reasonably supposed to be antigenically inert. The present study leaves the more provocative aspects of cellular immunity still to be answered and, indeed, probably raises more new questions than it answers existing ones. It does permit certain tentative conclusions to be drawn, however, and suggests further profitable experiments.

Pulmonary tuberculosis produced in mice under conditions simulating a natural infection (viz. with a tiny intranasal infecting dose) was profoundly modified by Triton used prophylactically, despite the failure of Triton to exhibit either bacteriostatic or bactericidal activity *in vitro*, thus confirming earlier reports to this effect. Reference to Fig. 2 shows that this effect was well established by about the 7th day and continued up to the onset of the immune phase. At this stage the activity of Triton merged smoothly into the altered immunological state and added nothing to it.

Furthermore, Triton given for the first time to mice as they converted to the allergic state appeared to add nothing to the existing, specifically acquired resistance. This observation confirms the findings of Kátó & Gözsy (1957) that Triton failed to increase the existing level of immunity in mice specifically immunized with BCG, and suggests the possible identity of the two states. Such a concept would imply that Triton might in some way be capable of inducing the same degree of enhancement of antituberculous activity as follows specific immunization, but that it does so very much more quickly. However, there is no indication at present of the existence of a mechanism which might allow this to take place. The absence

of allergy from the early part of the Triton-induced steady state may be unimportant, since by the time this work was undertaken the evidence against allergy *per se* playing any important role in immunity was already quite strong (Raffel, 1948; Gray, 1958, 1964). That the two phenomena are normally concurrent may mean no more than that they are both dependent on the same sort of stimuli, i.e. those arising from the products of lysed tubercle bacilli. If the action of Triton does actually stimulate an accelerated immune response, it is not only notably independent of allergy but also of the need for specific antigenic stimulation.

Such a supposition requires that the mechanisms governing cellular immunity be less precise than those of antibody production, allowing for possible induction by several apparently unrelated stimuli, some of which bear no apparent resemblance to known antigens, and there is certainly no proof at the present time that this is so. Another possible explanation is that specific cellular immunity does not exist at all, but that macrophages possess a latent, non-specific, bactericidal ability which is inducible by a variety of stimuli. Thus, there are many questions still to be answered.

Experiments are now under way to compare the relative speed of onset, efficiency and duration of cellular immunity produced by specific and non-specific means and to study the effects of Triton on the immune and steady states in an acute disease, viz. listerosis.

It is yet to be decided whether an animal in the steady state with respect to one disease is automatically in the same state with respect to all other intracellular infections as some of the recorded observations suggest. If this were so it would presuppose involvement of the entire macrophage system whenever cellular resistance is increased, and this, again, is inconsistent with what we know of specific immunological behaviour.

Whatever the nature of the steady state may be, it is clearly a dynamic compromise between host and parasite rather than a quiescent state in tuberculosis. Recently completed experiments (Cheers & Gray, to be published) have shown that alterations produced in the bacterial population of the steady state by chemotherapy and/or by superinfection are rapidly readjusted to the previous steady state at the earliest opportunity. The duration of this readjustment is now being examined.

SUMMARY

1. Non-specific stimulation of mice by a detergent lacking *in vitro* antibacterial activity, Triton WR 1339, produces a steady state in the course of experimental tuberculosis at about the same level as specific immunization, and does so independently of delayed allergy.

2. This effect is achieved in a much shorter time than is required by the normal immunological processes and the relative duration of enhanced resistance in each case is yet to be worked out.

3. Triton fails to enhance an existing, specifically acquired immunity.

4. There is no evidence so far that the mechanism involved is the same in both instances. If it were found to be the same, then the present concept of a specific cellular immunity would need to be revised.

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