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THE IMMUNOGENICITY OF PHAGOCYTOSED T4 BACTERIOPHAGE: CELL REPLACEMENT STUDIES WITH SPLENECTOMIZED AND IRRADIATED MICE

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SUMMARY

The role of phagocytosed antigen in the production of antibody to bacteriophage T4 has been studied. The ability of mice to give an antibody response to this antigen was first impaired either by splenectomy or by X-irradiation, and then restored by injection of syngeneic lymphoid cells given at various times relative to the injection of T4. In splenectomized animals administration of lymphoid cells had only a marginal effect on the severely depressed response to T4. It was concluded that the presence of an intact spleen is essential to the development of the normal immune response, and that circulating immunocompetent cells are unable to respond to circulating antigen or to antigen sequestered within the liver. On the other hand, in irradiated mice, there was a faster and more complete restoration of the anti-T4 response, confirming the ability of antigen localized within the spleen to stimulate competent cells. It was also found that the immunogenicity of T4 within this organ was not lost at a rate which corresponded to its gross breakdown but persisted without decrease for at least 48 hr. A similar observation was made for sheep red blood cells when this antigen was used in conjunction with T4.

INTRODUCTION

Evidence is steadily accumulating which implies that the uptake of certain antigens by phagocytic cells of the reticulo-endothelial system (RES) is an essential step in the initiation of antibody synthesis (Adler, Fishman & Dray, 1966; Ford, Gowans & McCullagh, 1966; Gottlieb, Glisin & Doty, 1967; Unanue & Askonas, 1968b; Mitchison, 1969). Although these experiments have been concerned with peritoneal macrophages, morphological and molecular studies suggest that other mononuclear phagocytes may act in a similar way (Ishizaka, Campbell & Ishizaka, 1960; Rolfe & Sinsheimer, 1965; Frei, Benacerraf & Thorbecke, 1965; Nossal *et al.*, 1966). Such evidence, however, is mainly circumstantial, and does not compare the relative activities of different components of the RES.

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It has previously been demonstrated that the catabolism of phagocytosed T4 bacteriophage may proceed at different rates in the mouse liver and spleen (Inchley, 1969). In view of this difference, the question arises as to whether the macrophages in these two sites possess a similar potential with respect to the induction of antibody production. The observation that little or no antibody was made in normal mice following the transfer of syngeneic Kupffer cells which had phagocytosed T4 *in vivo* suggested that they were incapable of effectively taking part in the induction of an immune response to this antigen (Inchley, 1969). This conclusion is in accord with the finding of Franzl (1962) that immunogenic fragments of sheep erythrocyte persisted within splenic macrophages, but disappeared rapidly from Kupffer cells.

The experiments described here represent a consideration of the ability of spleen macrophages and Kupffer cells to initiate antibody synthesis *in situ* following phagocytosis of T4. The capacity of mice to make antibody was first impaired by splenectomy or by sub-lethal X-irradiation, and in each case the restorative effect of normal syngeneic lymphoid cells was assessed as follows. In the first instance, splenectomized mice were given injections of spleen or lymph node cells, prior to a single immunizing dose of T4. Little or no improvement in the antibody response was detected, indicating that the depressant effect of splenectomy is attributable to the absence of a functionally intact spleen (with the opportunity for macrophage–lymphocyte interaction), rather than to a relative lymphocyte deficiency or loss of a particular cell category. Secondly, mice were injected with T4 24 hr after X-irradiation, and then given spleen cells at times up to 7 days later. The levels of antibody in these animals demonstrated the persistence of immunogenic material within host macrophages, and it was concluded that this was predominantly a function of antigen localized in the spleen, rather than that in the liver.

MATERIALS AND METHODS

Animals

Adult CBA mice were used which had been bred in the Department of Zoology, Edinburgh University. For each experiment, animals were of the same age and sex, with a weight range of 18–25 g.

Immunization

(1) *T4 bacteriophage.* Mice were immunized with 5×10^8 plaque-forming units (PFU) T4. The preparation of sera and assays for anti-T4 antibody were carried out as described previously (Inchley, 1969). All titres are expressed in terms of the phage inactivation constant '*K*' (Adams, 1959).

(2) *Sheep red blood cells (SRBC)* were washed three times and diluted to 20% (v/v) in isotonic saline. Mice were immunized with 0·1 ml of this suspension (10^8 cells). Haemagglutinin titrations were carried out using doubling dilutions of sera in 0·1 ml saline. Equal volumes of a 1% suspension of washed SRBC were added to each well. Incubation for 2 hr at 37°C was followed by standing overnight at 4°C before reading the titres.

Splenectomy

Splenectomy was performed under Avertin anaesthesia (0·1 ml i.p. of a 1:50 dilution in 10% ethanol/20 g body weight).

X-irradiation

Mice were exposed to 500 r whole body irradiation under conditions described elsewhere (Inchley, 1969). This dose was sufficient to suppress the immune response to both T4 and SRBC (Table 1), but had little effect on the overall RE clearance and degradation of antigen.

TABLE 1. The effect of 500 r whole body X-irradiation on the response of mice to 5×10^8 PFU T4 or 10^8 SRBC

T4 (serum *K*)				SRBC (agglutinin titre)			
Normal mice		Irradiated mice		Normal mice		Irradiated mice	
35·4	16·0	0·014	0·006	1280	640	10	< 5
26·4	15·4	0·014	0·006	1280	640	10	< 5
24·8	13·2	0·010	0·004	1280	640	10	< 5
21·2	12·8	0·010	0·004	640	640	5	< 5
		0·008	0·002	640	320	5	< 5
		0·006		640		< 5	< 5
						< 5	

Mice were bled 10 days after immunization. PFU, Plaque-forming units; SRBC, sheep red blood cells.

Preparation of cell suspension

(1) Spleen cell suspensions were prepared in medium 199 (Glaxo Laboratories) as described by Howard (1961). Cell mortality was 5–10% as measured by trypan blue exclusion. Vital staining with neutral red showed the number of macrophages to be 5–10% of the total nucleated cells.

(2) Lymph node cell suspensions. Axillary, brachial and inguinal nodes were pooled and dissociated in the same manner as the spleens. Isolated cells were washed once in medium 199 and injected i.v. in a volume of 0·5 ml.

RESULTS

Failure of cell replacement to restore the impaired ability of splenectomized mice to respond to intravenous T4

Three groups of six CBA mice were splenectomized and two of them received $7{\cdot}5 \times 10^7$ syngeneic spleen cells or $5{\cdot}0 \times 10^7$ lymph node cells i.v. 24 hr later. These two groups received a similar cell transfusion after a further 24 hr. All three groups, together with six normal CBA mice, were immunized on the following day with 5×10^8 T4 PFU i.v. Individual sera were obtained 4, 7, 11, 14, 21 and 47 days later and their phage inactivation activity assayed. The geometric mean response curves of these four groups are illustrated in Fig. 1. The untreated control group showed a rapid rise to reach a near-maximal level by day 11, followed by a well-maintained immune response throughout the 47-day period of observation. The splenectomized group showed a profound depression of this response; although

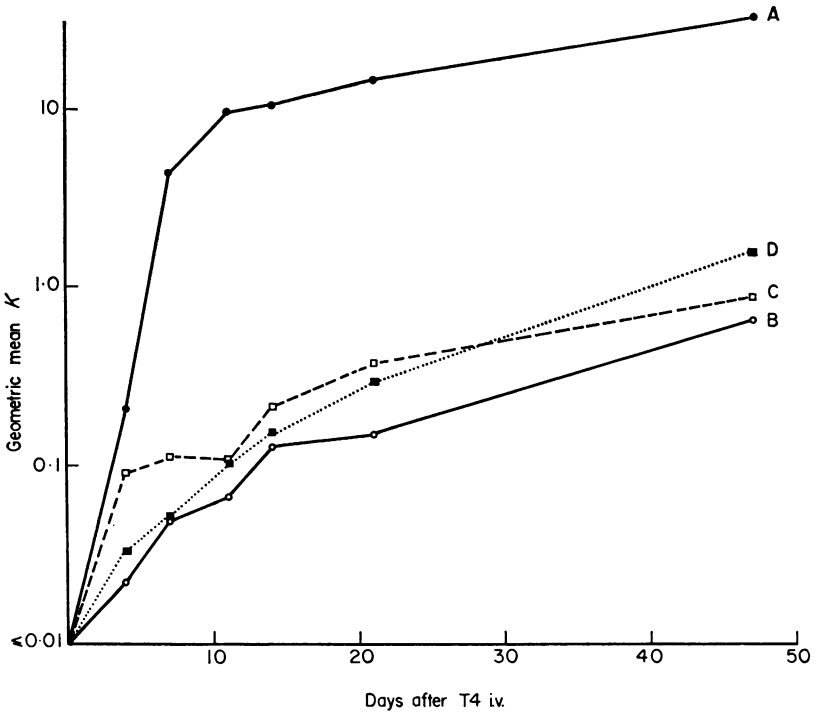

FIG. 1. The effect of two injections of lymphoid cells, given 48 and 24 hr before antigen, on the immune response to T4 in splenectomized mice. Group A, Untreated controls; B, splenectomized only; C, splenectomized and restored with spleen cells; D, splenectomized and restored with lymph node cells.

the K level rose steadily, it still remained two orders of magnitude below the control group by day 47. The splenectomized mice which had received prior injections of spleen or lymph node cell suspensions showed little or no improvement in their response. The mean K values for these groups were slightly higher throughout than in the splenectomized control group, but the differences recorded were relatively trivial.

In a second experiment, three groups of six CBA mice were splenectomized and two of these were given two cell 'replacement' injections as in the experiment shown in Fig. 1. After a further 110 days, 10^8 spleen cells and 5×10^7 lymph node cells were given i.v. to the appropriate groups, followed immediately by an i.v. injection of 5×10^8 PFU T4. The same dose of phage was given to the splenectomized control group and to an untreated group. The geometric mean 'K' response curves subsequently obtained are plotted in Fig. 2. Clearly none of the variations in experimental design incorporated here were at all effective in restoring the full capacity of response to the splenectomized mouse. Thus, antibody formation following an intravenous injection of T4 is largely dependent on the presence of an intact spleen. The effect of removal of this organ cannot be compensated for by replacing its constituent cells either before or simultaneously with antigen injection.

The immune response following transmission of spleen cells to irradiated mice injected with T4

The ability of phagocytosed antigen to stimulate immunocompetent cells at intervals after

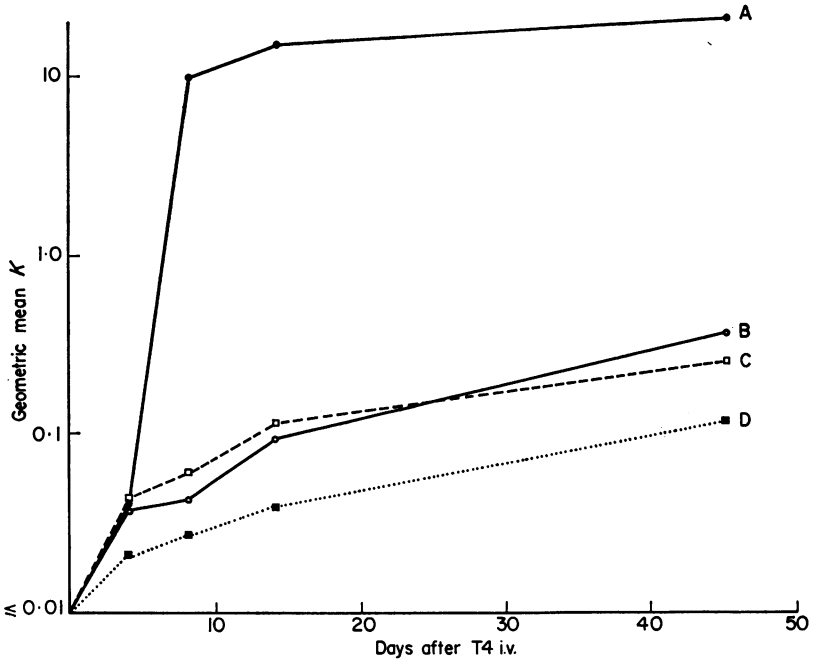

FIG. 2. The effect on the immune response to T4 in splenectomized mice of two injections of lymphoid cells given 24 hr apart and 110 days before antigen. A third injection of cells was given immediately prior to T4. Group A, Untreated controls; B, splenectomized only; C, splenectomized and restored with spleen cells; D, splenectomized and restored with lymph node cells.

immunization was further investigated by the transfer of syngeneic spleen cells into mice which had first been irradiated with 500 r and then injected with antigen 24 hr later. A single antibody determination was made for each recipient according to the time of replacement with spleen cells. For intervals up to 48 hr, sera were obtained 12 days after antigen (12–10 days after cell transfer). Recipients of cells at later times were bled 10 days after cell transfer. The initial exponential phase of the normal antibody response to T4 is completed by 7–10 days after immunization and thereafter there is relatively little increase in titre.

Six groups of five mice each were irradiated and injected with 5×10^8 PFU T4 24 hr later. Each group was given an i.p. injection of 10^8 spleen cells at times which varied from 12 hr before T4 until 48 hr afterwards. Following bleeding, the amount of antibody in individual sera was assayed and expressed in terms of the inactivation constant K (Table 2). Anti-T4 antibody was detected in all groups, but at relatively low levels when compared with the response elicited in normal animals by the same dose of phage (see Figs. 1 and 2). This was attributed partly to the route of administration of cells, as well as to the fact that the number of lymphocytes transferred was considerably less than the number assumed to have been damaged by irradiation. The most striking observation was that the extent of antibody formation was influenced very little by the time of spleen cell injection. Despite the rapid phagocytosis and breakdown of T4 during the first few hours after injection (Inchley, 1969), an immunogenic stimulus was still available to cells transferred at 48 hr and was little decreased from that available to cells given with or prior to T4.

TABLE 2. Antibody levels in irradiated CBA mice following the i.p. injection of 10^8 syngeneic spleen cells at various times after the i.v. injection of 5×10^8 PFU T4

	Time of cell transfer relative to T4 injection					
	−12 hr	0 hr	+6 hr	+12 hr	+24 hr	+48 hr
K	0·432	0·702	0·284	0·226	1·006	0·254
	0·370	0·256	0·250	0·212	0·584	0·212
	0·336	0·228	0·240	0·198	0·316	0·190
	0·306	0·226	0·232	0·176	0·212	0·146
	0·298	0·212	0·232	0·172	0·102	0·134
Geometric mean *K*	0·346	0·288	0·246	0·196	0·332	0·182

These findings were confirmed and amplified when spleen cells were given intravenously, and at times up to 7 days after injection of either T4 alone or T4 with 10^8 SRBC. The amounts of antibody formed in groups of five to ten mice in response to these antigens is given in Tables 3 and 4.

In both experiments the formation of anti-T4 antibody was generally superior to that obtained after intraperitoneal transfer of cells, and in most groups represented a less than ten-fold decrease from the response found in normal mice. This probably reflected an earlier and more substantial repopulation of the spleen following injection by this route. In addition, it was found that the ability of phagocytosed T4 (or some breakdown product) to induce lymphoid cells to make antibody persisted for at least 7 days. As was seen following i.p. injection of cells, there was no decrease in immunogenic activity during the first 48 hr. It is, therefore, apparent that the immunogenic stimulus was independent of the catabolism of the vast majority of ingested antigen during this period. However, when T4 was the only antigen, there was a marked decrease in its immunogenicity between 48 and 72 hr (Table 3). Antibody levels in mice given spleen cells 72 and 96 hr after T4 were ten times less than in those given an earlier injection of cells. This drop in T4 activity was much less apparent in

TABLE 3. Antibody levels in irradiated CBA mice following the i.v. injection of 10^8 syngeneic spleen cells at various times after the i.v. injection of 5×10^8 PFU T4

	Time of cell transfer relative to T4 injection						
	−12 hr	0 hr	+12 hr	+24 hr	+48 hr	+72 hr	+96 hr
K	2·570	1·584	4·270	2·394	3·548	0·224	1·914
	1·914	1·326	4·170	1·914	3·468	0·188	0·342
	0·204	1·236	2·278	1·768	3·424	0·150	0·178
	0·144	1·104	1·864	1·200	3·066	0·138	0·160
	died	0·164	0·188	1·124	1·748	0·132	0·160
					1·494	0·102	0·136
					1·406	0·096	0·134
					1·124	0·096	0·120
					1·006	0·092	0·070
					0·660	0·086	Died
Geometric mean *K*	0·348	0·862	1·705	1·615	1·800	0·124	0·198

TABLE 4. Antibody levels in irradiated CBA mice following the i.v. injection of 10^8 syngeneic spleen cells at various times after i.v. injection of 5×10^8 PFU T4 and 10^8 SRBC simultaneously

	Time of cell transfer relative to antigen injection					
	0 hr	+12 hr	+24 hr	+48 hr	+96 hr	+ 7days
Anti-T4	1·396	1·922	2·750	2·440	1·830	2·070
K	1·350	1·830	2·070	2·020	1·730	0·828
	1·350	1·786	1·830	1·786	1·350	0·828
	0·968	1·472	1·696	1·516	0·828	0·576
	0·688	1·310	1·396	1·226	0·750	Died
Geometric mean *K*	1·113	1·646	1·900	1·750	1·217	0·952
Anti-SRBC	640	320	640	320	80	80
Reciprocal titres	320	320	320	160	80	80
	160	320	320	160	40	40
	160	160	320	80	40	40
	160	80	80	40	20	Died
log-based mean	243	211	320	122	46	57

In each column the results are arranged in rank order only with respect to the level of either antibody.

animals given both antigens together. In these mice (Table 4) peak phage immunogenicity occurred at 24–48 hr. There followed a slight decrease so that the amount of anti-T4 antibody circulating 10 days after a 7 day transfer was only half that following transfer at 48 hr. The reason for the difference between these two sets of results is not clear, unless the rate of degradation of T4 was affected by the simultaneous disposal of ingested SRBC.

The response to SRBC, as measured by the haemagglutinin titres in recipient mice, showed similar features to those described for T4 (Table 4), and confirmed the persistence of antigenic activity. Antibody was again formed in animals given spleen cells as late as 7 days after antigen. Peak titres were found following the 24 hr transfer and thereafter there was a slow decrease in antibody levels with increasing intervals before administration of spleen cells. Since sera were obtained 10–12 days after lymphocyte replacement, these results probably represent a true drop in peak titre rather than the prolonged latent period observed by Ford (1968).

DISCUSSION

T4 bacteriophage is rapidly cleared from the circulation by the RES in both normal and irradiated mice (Inchley, 1969). It was found that the bulk of phage particles which are taken up by Kupffer cells are inactivated and degraded at a faster rate than is the relatively small amount phagocytosed by spleen macrophages. The same distinction was observed in normal and irradiated mice, although there was less initial phage localization in the spleen of the latter group. The results of transferring syngeneic Kupffer cells which had ingested T4 *in vivo* implied that a rapid loss of immunogenicity also occurred. The interpretation was favoured that this group of macrophages were incapable of playing any important role in the initiation of a primary immune response to this antigen.

The failure of cell replacement injections to effect any appreciable improvement in the early response of the splenectomized mouse to T4 suggests that lymphocyte deficiency alone is not a major factor in its depression. These results also indicate the relative inability of antigen-charged Kupffer cells *in situ* to transfer an immunogenic stimulus to circulating lymphocytes, as well as the apparent lack of importance of free circulating phage particles in this role. On the other hand, the slow and gradual rise in antibody titre which was observed in all splenectomized, T4-immunized mice suggests that *some* effective immunogenic material is persisting. Whether this is operating from within the liver or represents small amounts of antigen retained elsewhere is uncertain. Although long-persisting immunogenic fragments have been isolated from the liver (Garvey & Campbell, 1957), it remains doubtful whether any immunological function (such as maintenance of antibody production) can be assigned to Kupffer cells (Sterzl, 1959; Nossal, 1960).

The requirement for an anatomically intact spleen in this system emphasizes the importance of the T4 antigen which is phagocytosed by splenic macrophages in the induction of an immune response. Splenic lymphocytes themselves do not appear to represent any special, indispensable category. Indeed, Ford & Gowans (1967) recently showed that these cells are continually and rapidly migrating through the spleen, and that this appears to be important for effective induction via macrophage-localized antigen. As almost the whole early immune response to i.v. T4 is attributable to the spleen, the restorative effect of cell replacement in irradiated mice recorded here affords an *in vivo* measure of the persistence of immunogenic material within this organ. Essentially similar experiments have recently been reported by Ford (1968) using irradiated rats immunized with sheep erythrocytes and by Britton and his colleagues with a variety of antigens in mice (Britton & Celada, 1968; Britton, Wepsic & Möller, 1968). The present experiments do not shed any light on the question as to whether the immunogenic stimulus provided by the spleen macrophages consists of undegraded tail-protein antigen or antigen fragments (with or without complexing with RNA). The responses following a delay in spleen cell replacement implied that there was a progressive diminution after 48 hr in the effective amount of immunogenic material retained, although this was still detectable 1 week after injection.The previous studies on the fate of phagocytosed T4 (Inchley, 1969) indicate that it is rapidly inactivated and broken down, but part of the constituent antigens may well be localized in cytoplasmic organelles and protected from catabolism, as suggested by the work of Unanue & Askones (1968a), Kölsch & Mitchison (1968) and Uhr & Weissmann (1965). This could well explain why immunogenicity does not appear to be lost at a rate proportional to phage destruction. It is not possible to say from these *in vivo* experiments whether the persisting immunogenic stimulus in the spleen becomes continuously available almost immediately following phagocytosis of bacteriophage, although *in vitro* studies with peritoneal exudate cells suggest that this would be the case (Adler *et al.*, 1966). The fact that adoptive immunity could not be transferred with spleen cells until 48 hr after i.v. injection of T4 (Inchley, unpublished) need not necessarily reflect delayed appearance of the stimulus, but be attributable to the time needed for effective contact between macrophages and lymphocytes or for migration of the latter cells through the splenic framework.

In contrast with the distinction between liver and spleen macrophages with regard to the fate of T4 phage as antigen, it has been found recently that the immunogenicity of bovine serum albumin is increased as effectively after uptake by Kupffer cells as it is by alveolar and peritoneal macrophages (Mitchison & Howard, unpublished). It appears that results of

studying the immunogenicity of macrophage-associated material are affected by the antigen employed as well as by the class of macrophage. These and other differences recorded suggest that the role of macrophages in the induction of an immune response is dependent on more than one mechanism. Firstly, Gallily & Feldman (1967) and Mitchison (1969) have found that the highly immunogenic activity of macrophage-enclosed *Shigella dysenteriae* antigen or BSA, respectively, was lost when the cells had been previously irradiated with doses exceeding 550 r. On the other hand, Unanue & Askonas (1968b) could detect no comparable effect using haemocyanin. Similarly, the present results and those of Ford (1968) imply that T4 and sheep erythrocytes were effectively immunogenic following phagocytosis by irradiated splenic macrophages *in vivo*. Secondly, the decay in immunogenicity of macrophage-enclosed antigens clearly varies considerably according to the antigen used. In the case of human serum albumin (HSA) (Britton & Celada, 1968) it may be very short indeed whereas the immunogenic activity of *E. coli* lypopolysaccharide has been shown to persist for several weeks (Britton *et al.*, 1968). Although the macrophage may function differently with regard to poorly immunogenic soluble proteins such as HSA and highly immunogenic particles like T4, it would be premature to elaborate this argument in view of apparent inconsistencies in the data currently available.

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