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Studies on Phagocytosis

I. ANTIGEN CLEARANCE STUDIES IN RABBITS

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Summary. Clearance of T_1 or T_2 bacteriophage from the circulation of rabbits takes place in two phases; a rapid first phase and a slower second phase. Secondary stimulation of rabbits with a similar dose of phage to the primary dose results in a more rapid clearance of bacteriophage particles from the circulation. This increased rate of clearance can be demonstrated prior to the production of any detectable humoral antibody. The enhanced secondary clearance appears to be specific for the particle concerned, and does not occur using non-antigenic carbon particles.

INTRODUCTION

From numerous studies on the clearance of antigenic and non-antigenic particles from the circulation, Benacerraf, Biozzi, Halpern and Stiffel (1957) have shown that the clearance of uniformly sized colloidal particles from the circulation can be expressed as an exponential function, and that a phagocytic index, K, being a measure of the rate of clearance of the particles, and thereby of the rate of phagocytosis by cells, can be calculated from the equation:

$$K = \frac{\log \text{conc. } a - \log \text{conc. } b}{t_a - t_b}$$

where t_a and t_b are the times at which concentration a and b occur.

Benacerraf *et al.* (1957) demonstrated that when the amounts of the colloid injected are larger than a critical dose, the value of K becomes dose dependent, so that $K \times D =$ constant, where D is the dose injected.

The rate of clearance appears to differ for each particle used. In general the bigger the particle, the faster its rate of removal (Dobson, Gofman, Jones, Kelly and Walker, 1949). Using a preparation containing particles of varying sizes, larger particles are cleared more quickly than smaller ones, and a biphasic clearance curve results when a preparation containing two sizes of particle is injected into the circulation of an animal.

This work was undertaken as part of a phylogenetic study on the clearance of antigens from the circulation of animals, and their subsequent humoral responses. In this paper only work on the rabbit is presented; the results of clearance studies on lower vertebrates and invertebrates will be presented elsewhere.

MATERIALS AND METHODS

Animals

Rabbits used were both male and female of the local outbred strain and were tested for the presence of neutralizing antibody to the bacteriophage used, prior to clearance studies.

Antigens

(1) Bacteriophage T_1, propagating strain *E. coli* B.

(2) Bacteriophage T_6, propagating strain *E. coli* B.

(3) Bacteriophage T_2, propagating strain *E. coli* B.

All phages were obtained from the Bacteriology Department culture collection, Manchester University.

(4) A suspension of carbon in fish glue (Ink C/1432a), commercially available from Gunther Wagner, Hanover, Germany, containing approximately 100 mg carbon/ml. The ink was injected into the animals in 2 per cent gelatin to ensure stability of the preparation.

(5) Endotoxin prepared from overnight broth cultures of *E. coli* B. The bacteria were disintegrated in a Mickel shaker and the broth membrane filtered and checked for sterility before use. Such endotoxin preparations were assumed to contain at least as much endotoxin as the phage preparations used.

Bacteriophage assay

(1) *Assay of plasma samples for bacteriophage.* The following pour plate technique was employed. The plasma sample was diluted appropriately in nutrient broth. The plasma dilution (0·1 ml) was pipetted into 5 ml of 0·7 per cent nutrient agar, previously melted and cooled to 50°, and to which 0·2 ml of a 4-hour broth culture of *E. coli B* had been added. The contents of the tubes were thoroughly mixed by rolling before being poured evenly over the surface of a dried 1 per cent nutrient agar plate. After overnight incubation at 37° the number of bacteriophage plaques formed could be counted. The dilution giving between 20 and 60 plaques per plate was then re-assayed in quadruplicate.

(2) *Assay of plasma or serum for neutralizing antibody to phage.* A dilution of phage containing 4×10^2 phage p.f.u./ml was made in nutrient broth. Equal volumes of rabbit serum or plasma, and of the phage dilution were incubated together at 37° for 3 hours. A control tube containing equal quantities of nutrient broth and of phage dilution was included in all experiments.

After 3 hours incubation 0·1 ml amounts of the bacteriophage–serum mixture was assayed as previously described.

It was found that a standard error of ± 10 plaques was significant when counting 4×40 bacteriophage plaques on quadruplicate plates. Animals whose sera showed a 25 per cent reduction in phage plaques when tested for neutralizing antibody prior to clearance experiments were not used in this study.

Carbon assay

Heparinized blood samples were assayed for residual carbon by lysing 0·05 ml of whole blood in 4·95 ml 0·1 per cent Na_2CO_3. The optical density of the sample was then read at 650 mμ on a Unicam S.P. 600 spectrophotometer. The blank used was 0·05 ml heparinized blood taken immediately before the injection of carbon, and lysed in 4·95 ml 0·1 per cent Na_2CO_3.

Experimental procedure

In all experiments rabbits were injected intravenously into an ear vein and blood samples were obtained from the opposite ear vein. Blood samples were taken before every injection and checked for the presence of neutralizing antibody to bacteriophage. Heparin

was used as the anticoagulant in all experiments. All injections of bacteriophage were given in 1 ml amounts in nutrient broth.

(1) *Phage clearance.* Twenty-one rabbits were given intravenous doses of either T_1 or T_2 phage and clearance followed at intervals up to 120 minutes subsequently. The clearance of repeat doses was investigated at intervals of from 24 hours to 14 days after the primary dose.

(2) *Carbon clearance.* Three rabbits received 250 mg carbon in 2 per cent gelatin intravenously. Blood samples were obtained immediately before injection and at 0, 5, 10, 15, 20, 30, 40, 50 and 60 minutes subsequently. Forty-eight hours later the procedure was repeated. Forty-eight hours after the secondary dose, one rabbit received a tertiary dose and clearance was followed as before.

(3) *Antigenic specificity of response.* The clearance of T_1 phage was investigated as previously described 48 hours after the administration of: (a) T_2 phage, (b) T_6 phage, and (c) *E. coli B* endotoxin.

RESULTS

Bacteriophage clearance curves were constructed by plotting $\log_e$ concentration of phage against time. The phagocytic indices (K values) have been calculated in accordance with the equation of Benacerraf *et al.* (1957).

PHAGE CLEARANCE

(a) *Time interval between doses of T_1 phage*

The phagocytic indices of eleven rabbits given doses of T_1 phage and T_2 phage at differing time intervals are shown in Table 1. The clearance curves are shown in Figs. 1–6.

Clearance of the primary dose of bacteriophage from the circulation of each rabbit

TABLE 1

RELATION OF PHAGOCYTIC INDEX TO TIME INTERVAL BETWEEN DOSES

No. of rabbits	Dose	Time interval between doses (days)	1° K*	Average 1° K	2° K*	Average 2° K
1	4×10^6 T_1 phage	14	0·0829		1·85	
1	4×10^9 T_1 phage	14	0·055		1·25	
2	4×10^9 T_1 phage	2	0·0827 0·0216	0·0522	0·263 0·1824	0·2227
2	4×10^9 T_1 phage	1	0·1118 0·1176	0·1147	0·181 0·109	0·145
2	4×10^9 T_2 phage	2	0·08005 0·08812	0·0841	1·601 0·6297	1·1154
3	4×10^9 T_2 phage	1	0·0325 0·1269 0·06	0·0731	0·061 0·1555 0·176	0·1308
3	250 mg carbon	2	0·051 0·11 0·096	0·08567	0·037 0·067 0·096	0·0667

* 1° K, primary phagocytic idex; 2° K, secondary phagocytic index.

can be seen to have occurred in two phases, a rapid first phase, and a slower second phase. The rate of clearance of both fast and slow phases can be plotted along straight lines.

The phagocytic indices have been calculated from the rapid initial phase of clearance in all cases.

The clearance of the secondary dose of bacteriophage T_1 injected 14 days after the primary dose was very rapid in both rabbits, and reached zero after approximately 15 minutes (see Fig. 1). Both rabbits were found to have formed neutralizing antibody by

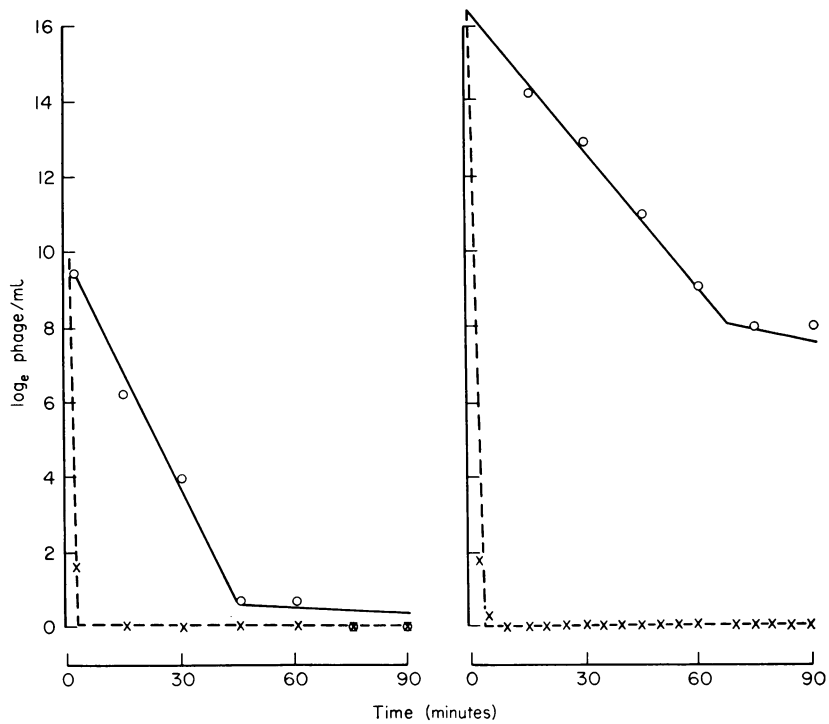

FIG. 1. Primary (○) and secondary (×) clearance curves of T_1 phage with a 14-day interval.

this time and the very rapid clearance was probably mediated by the opsonic activity of the specific antibody causing increased uptake of phage by the phagocytic cells of the rabbits, supplemented by some neutralization of the phage.

The clearance curves of doses 4×10^9 T_1 phage given 2 days after the primary dose of T_1 phage are shown in Fig. 2. The K values are given in Table 1. It can be seen that 48 hours after the initial dose the rates of clearance of the secondary dose, especially in the fast initial rates of clearance, were increased. No neutralizing antibody was detected in plasma samples taken immediately before the secondary dose.

Clearance of primary and secondary doses of 4×10^9 T_1 phage from two rabbits with a 24-hour interval between doses are shown in Fig. 3. The K values are given in Table 1. In one rabbit no alteration in the rate of clearance was detected, but in the other rabbit the secondary clearance was more rapid. No neutralizing antibody was detected in plasma samples taken before the administration of the secondary doses of phage.

Fig. 4 illustrates the rates of clearance of primary and secondary doses of T_2 phage from two rabbits injected with 4×10^9 T_2 phage particles with a time interval of 48 hours between primary and secondary doses. The K values are given in Table 1. It is apparent

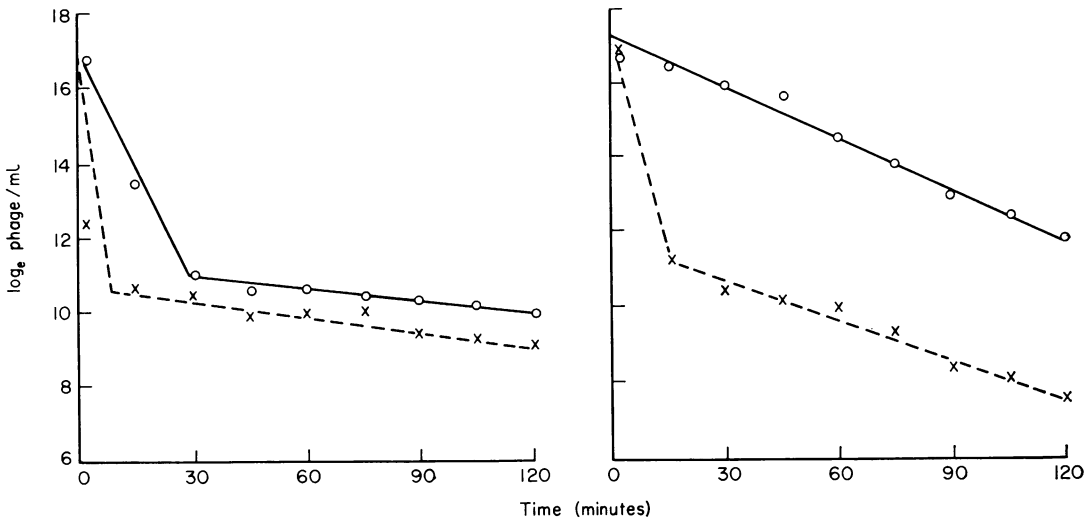

FIG. 2. Primary (○) and secondary (×) clearance curves of T_1 phage with a 2-day interval.

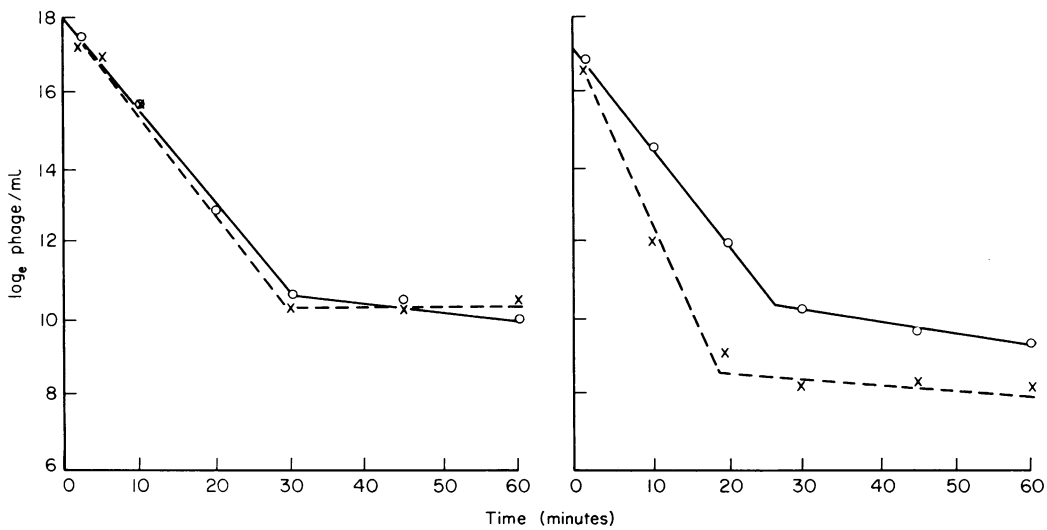

FIG. 3. Primary (○) and secondary (×) clearance curves of T_1 phage with a 24-hour interval.

that the clearance is similar to that for T_1 phage, being biphasic. The rate of clearance of the secondary dose is greatly increased when compared with that of the primary dose but in these two rabbits neutralizing antibody to T_2 phage was detected in plasma taken from both rabbits immediately before the second injection.

The rates of clearance of primary and secondary doses of 4×10^9 T_2 phage in two rabbits with a 24-hour time interval between doses is shown in Fig. 5. The K values are given in Table 1. The secondary clearance rates are again faster than the primary, but in neither rabbit was antibody to T_2 phage detectable before the injection of the second dose of bacteriophage.

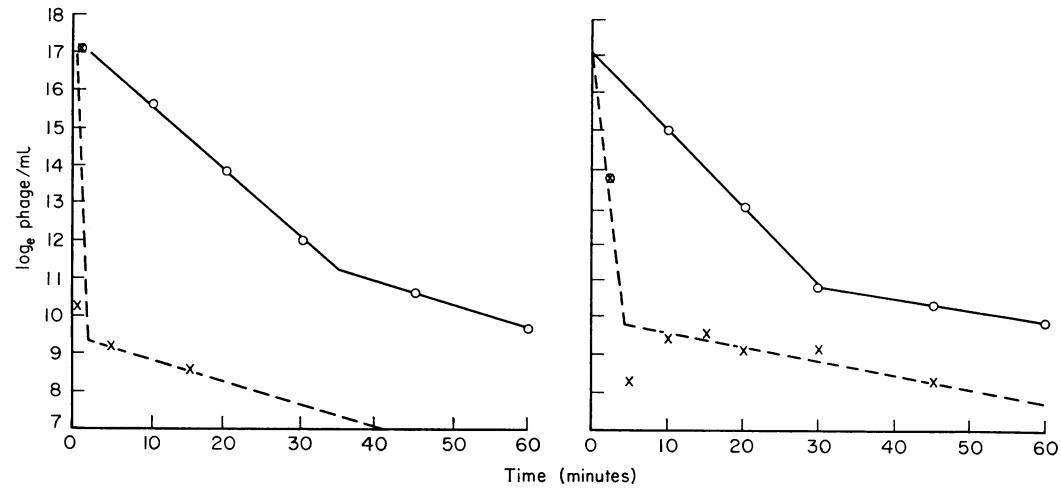

FIG. 4. Primary (○) and secondary (×) clearance curves of T_2 phage with a 48-hour interval.

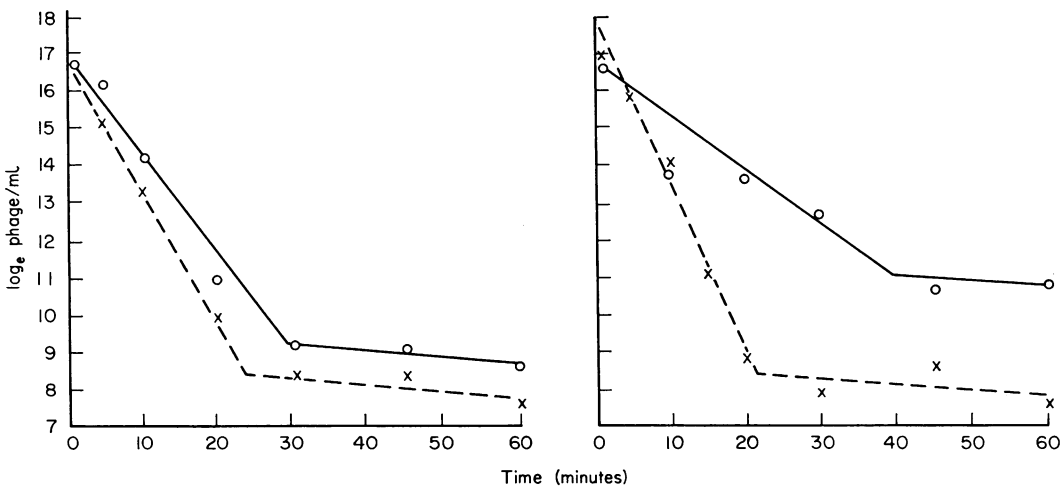

FIG. 5. Primary (×) and secondary (○) clearance curves of T_2 phage with a 24-hour interval.

(b) *Variation in dose of T_1 phage*

Fig. 6 illustrates the rate of clearance of doses of 4×10^{10}, 4×10^9, 4×10^8, 4×10^7 and 4×10^6 T_1 phage particles in five rabbits. The K values are shown in Table 2. From the average value of K for the different doses it would appear that there is a slight trend to a fall in K as the dose of phage particles increases.

From all the clearance curves, both of T_1 and T_2 phage, it is apparent that the point at which the rate of clearance becomes abruptly slower occurs when approximately 0·1 per cent of the injected dose remains in the circulation.

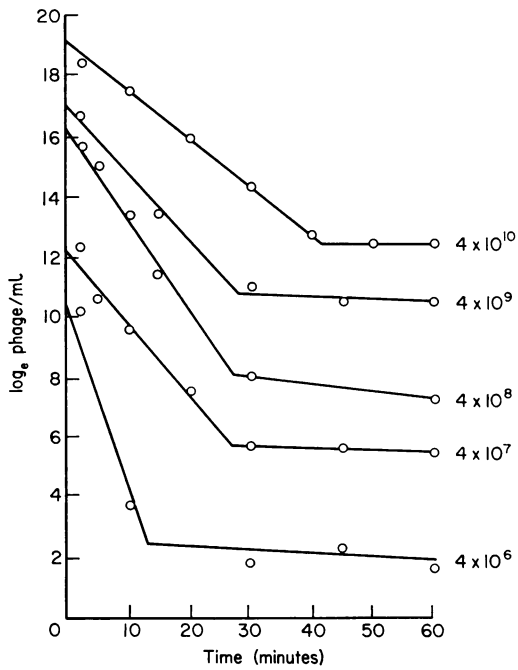

FIG. 6. To illustrate the rates of clearance of doses of T_1 phage from 4×10^{10} to 4×10^{6} particles.

TABLE 2

RELATION OF PHAGOCYTIC INDEX TO DOSE OF PHAGE INJECTED WITH A 48-HOUR INTERVAL BETWEEN DOSES

No. of rabbits	Dose	1° *K**	Average 1° *K*	2° *K**	Average 2° *K*
1	1×10^{10} T_1 phage	0·07053			
8	4×10^{9} T_1 phage	0·055 0·0827 0·02156 0·1118 0·1176 0·1004 0·06537 0·1127	0·08339 Standard error 0·03705	0·2138 0·1807 0·181	0·1918
1	4×10^{8} T_1 phage	0·1246			
2	4×10^{7} T_1 phage	0·10243 0·06225	0·08239	0·8239 0·431	0·6275
3	4×10^{6} T_1 phage	0·0688 0·276 0·2621	0·2023	0·134 0·4367 0·372	0·3142

* Abbreviations as in Table 1.

CARBON CLEARANCE

Fig. 7 illustrates examples of the clearance of primary, secondary and tertiary doses of 250 mg carbon in 2 per cent gelatin with a 48-hour interval between doses. It is apparent that the clearance of carbon does not follow the same exponential function throughout the duration of the experiment. The time at which a decrease in rate of clearance occurred differed considerably for each rabbit, but was similar for both primary and secondary responses in the same rabbit.

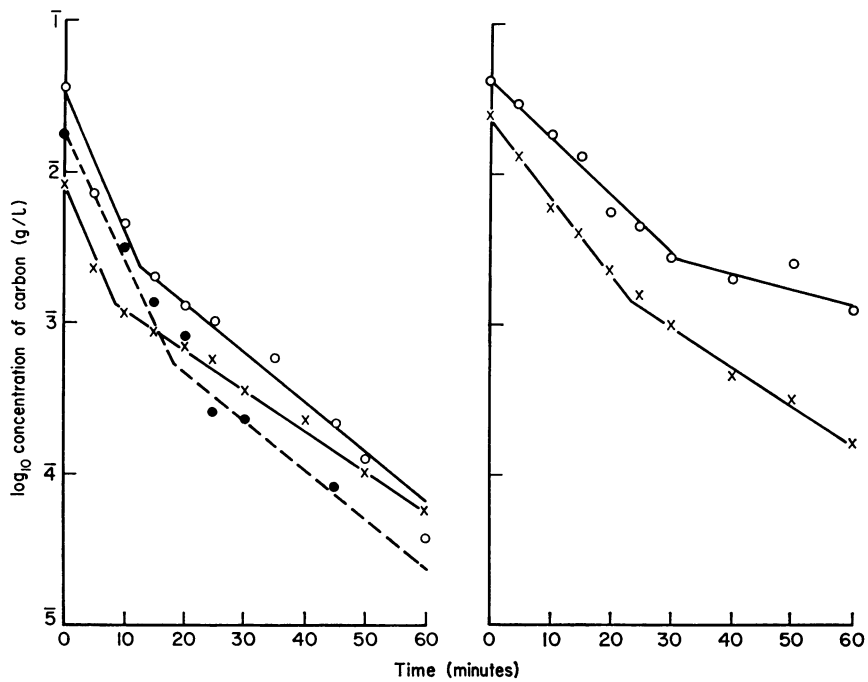

FIG. 7. Primary (×), secondary (○) and tertiary (●) clearance curves of 250 mg carbon administered at 48-hour intervals.

It can be seen from Table 1 that the K values calculated from the initial fast rate of clearance are not increased after secondary administration of the animals with carbon. However, in two rabbits the rate of clearance of the slower phase of the secondary response was increased.

SPECIFICITY OF RESPONSE

(a) T_2 *phage*

Fig. 8 illustrates one example of the clearance of 4×10^9 T_1 phage 48 hours and 72 hours after the injection of 4×10^9 T_2 phage. The K values are shown in Table 3.

(b) T_6 *phage*

Fig. 9 illustrates the clearance of 4×10^9 T_1 phage from two rabbits 48 and 72 hours after the injection of 4×10^9 T_6 phage. The K values are given in Table 3.

The clearance of T_6 phage from the circulation was not followed, since preliminary experiments had demonstrated that such studies gave unreliable results, due to the instability of the bacteriophage.

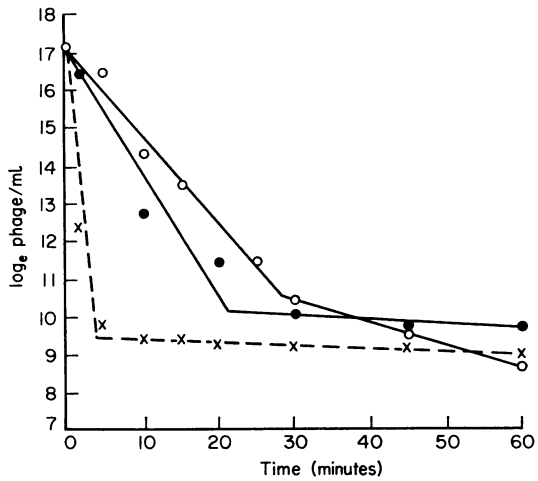

FIG. 8. Illustrates the clearance of 4×10^9 T_1 phage 48 and 72 hours after clearance of a dose of 4×10^9 T_2 phage. ●, Clearance of 4×10^9 T_2 phage; ○, clearance of 4×10^9 T_1 phage (primary); ×, clearance of 4×10^9 T_1 phage (secondary).

TABLE 3

K VALUES* FOR PRIMARY AND SECONDARY RESPONSES TO T_1 PHAGE 48 HOURS AFTER THE INJECTION OF ANTIGENICALLY UNRELATED MATERIAL

No. of rabbits	Dose of antigenically unrelated material	Dose of T_1 phage	1° *K** to T_1 phage	2° *K** to T_1 phage
2	4×10^9 T_2 phage	4×10^9	0·1038 0·09041 Average 0·0971	0·7622 0·6312 Average 0·6967
2	4×10^9 T_6 phage	4×10^9	0·1233 0·1463 Average 0·1348	
2	1 ml *E. coli B* endotoxin	4×10^9	0·1012 0·0933 Average 0·09725	0·4841 0·2844 Average 0·3834

* Abbreviations as in Table 1.

(c) *Endotoxin*

The clearance of 4×10^9 T_1 bacteriophage from two rabbits 48 hours and 72 hours after the administration of 1 ml *E. coli* B endotoxin, is shown in Fig. 10. The *K* values are given in Table 3.

From Table 3 it can be seen that the average *K* values for primary response to T_1 phage in experiments (a), (b) and (c) do not differ significantly from the average *K* value for a normal primary response to T_1 phage, shown in Table 2.

The *K* values for the clearance of secondary doses of T_1 phage for all the rabbits in experiments (a), (b) and (c) appear to be increased for the normal *K* value for a secondary

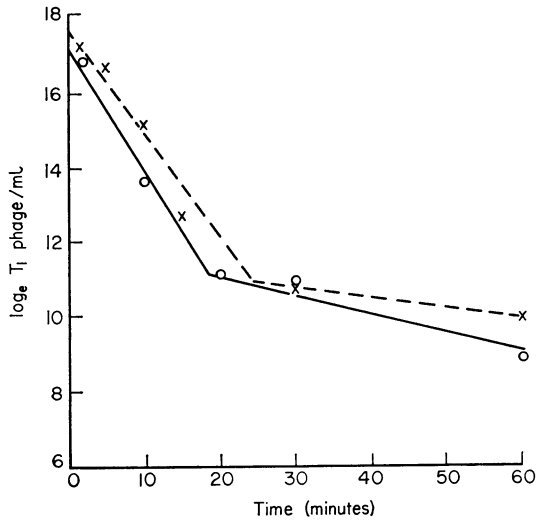

FIG. 9. Illustrates clearance studies of T_1 phage in two rabbits, which received respectively 4×10^9 T_6 phage, 48 (×) and 72 (○) hours previously.

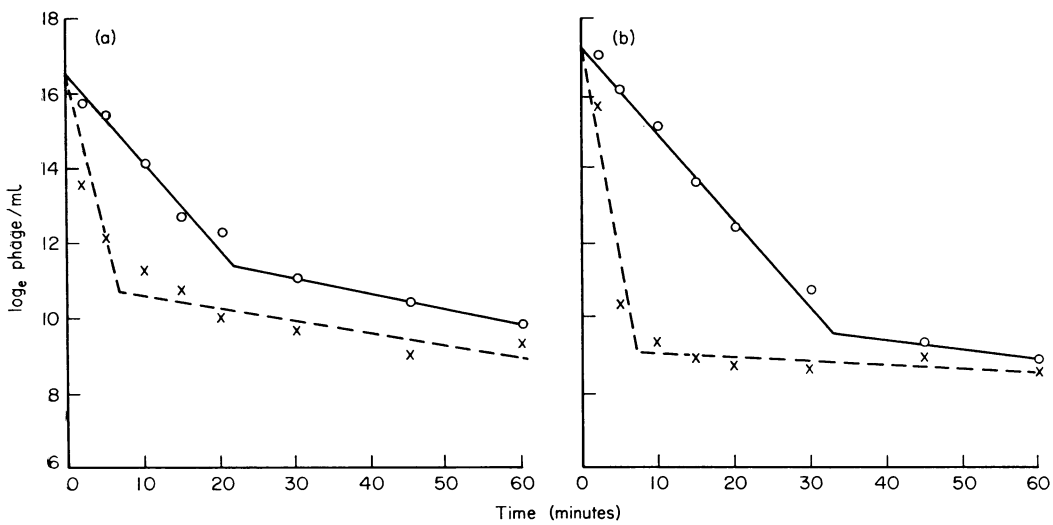

FIG. 10. Illustrates primary (○) and secondary (×) T_1 clearance curves 48 hours apart. (a) From a rabbit which received *E. coli* endotoxin 48 hours before primary T_1 clearance, and (b) from a rabbit in which the interval between endotoxin and primary clearance was 72 hours.

response to 4×10^9 T_1 phage. It was found that one rabbit in the group given T_2 phage had formed a very low level of antibody, detectable at a serum dilution of 1:2. No neutralizing antibody to T_1 phage was detected in any of the other five rabbits.

The form of the secondary clearance curves of T_1 phage in both rabbits given endotoxin appear to differ from the normal in that the change from fast to slow phases of clearance is far more gradual than is usual.

DISCUSSION

The results of clearance studies on T_1 and T_2 phage clearly indicate that an increased secondary rate of clearance of phage particles can be shown to occur before the production of any detectable humoral antibody. The effect can be demonstrated as early as 24 hours after the primary immunizing dose.

It has also been shown that this increased rate of clearance is not caused by the non-specific effects of endotoxin in the phage preparation nor is it induced by the previous administration of antigenically unrelated particles. It appears to be specific for the particles concerned, and only occurs if antigenic particles are used.

Biozzi, Benacerraf and Halpern (1955) have reported that the phagocytic activity of the RES of mice for *S. typhi* or its endotoxin is increased 48 hours after the administration of an intravenous injection of *S. typhi* or its endotoxin. They studied the activity of the RES by following the rate of clearance of doses of carbon injected simultaneously with the doses of bacteria or toxin, and showed a depression in RES activity lasting for approximately 24 hours after the primary injection, and subsequently increasing above normal. These workers did not investigate the sera of the mice for antibody to *S. typhi* prior to the clearance studies, and it is our experience that the majority of animals have some low titre antibody to *S. typhi* in their sera prior to any known stimulation with the organism. Such antibody would in any case cause an accelerated clearance of bacteria.

The same group of workers (Biozzi, Halpern, Benacerraf and Stiffel, 1957) reported a similar increase in the activity of the RES after a transient period of depression, following blockade of the RES by saccharated iron oxide. No increase in phagocytic activity was noted following blockage of the RES by carbon particles (Biozzi, Benacerraf and Halpern, 1953, 1957). They also noted that injection of a denatured albumin–globulin complex caused a rise in the phagocytic activity of the RES towards carbon particles, but found that the injection of mice with dead staphylococci had no effect on RES activity. The rise in phagocytic index was accompanied by an enlargement in the size of the liver and spleen of the animals used, presumably due to the multiplication of the phagocytic cells of these organs.

An increase in the rate of clearance of T_4 phage from the circulation of rabbits was noted by Jerne (1960). He found that if primary and secondary injections were 24 hours apart, the rate of elimination was the same. At 48 hours the rate was slightly faster, and at 72 hours the immune elimination was greatly increased. He does not mention whether he checked the plasma for antibody prior to the injection.

Apart from the above reports of Biozzi *et al.* and Jerne there appears to be no reference in the literature to the phenomenon of increased secondary clearance of antigen prior to the production of detectable specific antibody.

The results of Biozzi *et al.* apparently show that the intravenous injection of large amounts of particulate matter cause a non-specific increase in the phagocytic activity of the RES. These results are obviously in conflict with those reported in this paper.

Our results however, agree with those of Herring, Herion, Walker and Palmer (1963) who found that although the clearance of ^{51}Cr-labelled endotoxin was faster in febrile tolerant rabbits than in normal rabbits, the clearance of non-antigenic $^{51}CrCl_3$ was no faster in rabbits tolerant to endotoxin than it was in normal rabbits.

The work of Herring *et al.* (1963) does not agree with that of Biozzi *et al.* in that in one case the non-antigenic particle carbon is cleared more rapidly from an animal previously

injected with endotoxin; whereas in the other case, there is apparently no increase in the rate of clearance of the non-antigenic particle $^{51}CrCl_3$ after the injection of endotoxin, despite the fact that increased uptake of endotoxin occurred at this time.

A point of difference between the two reports above is that there was no primary stimulation with $^{51}CrCl_3$ in the work of Herring *et al.* (1963). Biozzi *et al.* reported that although no increase in phagocytic index followed the injection of a blocking dose of carbon particles, an injection of saccharated iron oxide did cause a rise in phagocytic index. Whether an initial injection of $^{51}CrCl_3$ would have caused an increase in the rate of clearance of a second dose of $^{51}CrCl_3$ is a matter for speculation.

The only other difference between these two reports is that in one case endotoxin and non-antigenic particles were injected simultaneously, whereas in the other report they were injected separately.

It would seem likely that Biozzi *et al.* were detecting the augmentation of phagocytic activity of the RES known to be produced by endotoxin, and that the differences between their work and that of Herring *et al.* (1963) was due to differences in the preparations used. In the experiments reported here however, the effect of endotoxin has been excluded by the use of controls, which would contain at least as much, if not more, endotoxin than in the diluted phage preparations used.

Another point which should be noted is that the phage preparations used in these studies represent a considerably smaller dose of particulate material than those used by Biozzi *et al.* Their experiments were carried out in the range of dose of particulates where $K \times D$ is a constant value. This relation did not hold for the experiments reported in this paper and may be a contributing factor to the difference in results of the specificity of the response.

The reason for the biphasic clearance of phage particles seen in these experiments is not clear. That the initial rapid phase of clearance reduces the number of phage particles to a consistent percentage, regardless of the initial dose, is also suggested by the work of Jerne (1960) and Schiltz and Neva (1965) using T_4 and T_2 phages, respectively. The results also agree with those of Biozzi, Howard, Halpern, Stiffel and Mouton (1960) who observed this phenomenon when studying the blood clearance of isotopically labelled *Salmonella enteritidis* in mice.

These observations might be explained on the basis of the work of Dobson *et al.* (1949) and Dobson (1957). Their work on the intravenous injection of colloids into dogs and rabbits demonstrated that if the colloid contained particles of heterogeneous sizes a biphasic clearance curve was obtained, having a fast initial phase and a slow secondary phase. When blood containing the slowly disappearing fraction was injected into another animal, these particles continue to disappear slowly, thus indicating that this fraction was not efficiently phagocytosed. It was found that the rapidly disappearing component consisted of large sized particles, which, after clearance, could be found primarily in the liver and spleen, whereas the colloidal particles were found in relatively large amounts in the bone marrow.

Thus, if heterogeneous sizes of phage particles existed in the preparation approximately 0·1 per cent of them being smaller than the rest, then the slow rate of clearance would commence when all the large particles had been phagocytosed and only the small particles remain. However, there is no evidence to indicate that bacteriophage particles, such as the ones used in this study, contain particles of widely differing sizes, nor do they appear to contain aggregates of particles. That the slowly disappearing fraction consists of frag-

ments of phage particles is not possible since such fragments would not form plaques on assay. Hence it must suffice to say that a proportion of the phage particles are not efficiently phagocytosed, although the explanation of this phenomenon is not clear.

In this work using bacteriophage no clear inverse relationship between phagocytic index, K, and dose of phage could be established, nor was it established by Biozzi *et al.* (1953) using bacteria, in contrast to their findings on the clearance of non-antigenic colloids. It may be possible that the doses of phage used were not sufficiently high for this phenomenon to become apparent, since Solomon (1966), working on the clearance of goat erythrocytes from chickens, found that $K \times D$ = constant only held within the dose range of 1×10^8 to $1{\cdot}3 \times 10^{10}$ erythrocytes/ml.

The failure to detect any neutralizing antibody to T_1 phage at a time when an increased rate of clearance of phage from the circulation could be demonstrated, implies that the immune elimination of phage is mediated by a purely cellular means.

ACKNOWLEDGMENT

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