ANTIBODY FORMATION*

IV. Formation of Rapidly and Slowly Sedimenting Antibodies and Immunological Memory to Bacteriophage ϕX 174

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(Received for publication, November 20, 1962)

Following the injection of any one of various antigens into humans (1, 2) and rabbits (3–5), rapidly sedimenting antibody molecules (19S) first appear in the circulation, and later are replaced by molecules of 7S sedimentation constant. Similar results were obtained after injection of bacteriophage ϕX 174 into guinea pigs (6), newborn humans (7), sheep embryos (8), and several non-mammalian vertebrates (9), indicating the general occurrence of this sequence in the immune response.

Earlier studies of anti-ϕX formation in guinea pigs indicated certain similarities between the kinetics of primary 19S and secondary 7S antibody formations (6). The purpose of this study was to investigate the decline of 19S antibody formation, the primary 7S antibody response, and the relationship between the two responses. The results indicate that primary 7S antibody formation lasts for months and leads to preparation for a secondary 7S antibody response (immunological memory). In contrast, 19S antibody formation is relatively short-lived and in these studies did not lead to persisting immunological memory. It has also been shown that antigen can regulate the relative rate of antibody formation and the duration of rapidly sedimenting antibody synthesis, suggesting that the amount of antigen in immunologically competent cells may serve as a major control mechanism for several immunological functions.

Materials and Methods

Phage.—Two preparations of bacteriophage ϕX 174 were used. The first, grown in *E. coli* strain C on glycerol–casamino acid media (10) and purified by the method previously described (6), contained 4.5×10^{11} plaque-forming particles/ml. A second stock of ϕX, kindly

* Aided by grants from the National Institutes of Health, United States Public Health Service (E. 1821 (C-4)). This work was conducted in part under the sponsorship of the Commission on Immunization, Armed Forces Epidemiological Board, and was supported in part by the office of the Surgeon General, Department of the Army, Washington, D. C.

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supplied by Dr. R. L. Sinsheimer, contained 9×10^{13} particles/ml, of which 2.4×10^{13}/ml were plaque-formers. This latter preparation was slightly less immunogenic than the first, as shown by comparison of antibody responses after injection of guinea pigs with equal quantities of plaque-forming phage. Therefore, where major differences in responses to these two preparations were observed, Sinsheimer's ϕX will be designated ϕX (A) and the other stock designated ϕX (B).

T_2 bacteriophage, containing 10^{13} plaque-forming particles/ml, was prepared by the method described in Adams (11).

In this paper the number of phage particles will refer only to the plaque-formers.

Immunization.—Dilutions of phage in saline were injected intravenously into the hind leg of 400 gm Hartley guinea pigs or into the ear of 2 kg rabbits. Several guinea pigs were injected intraperitoneally with 10 μg of endotoxin (Difco *Salmonella enteritidis* lipopolysaccharide control No. 116644) contained in 0.1 ml of saline.

At intervals after immunization, guinea pigs were bled from the retro-orbital venous plexus using capillary pipettes, and rabbits from the ear vein.

Antibody Determinations.—The assay for antibody was carried out by the method described by Adams (11). The inactivation of phage by antibody follows first-order kinetics described by the relationship $ln(P_t/P_0) = kt/D$. P_0 is the phage assay at zero time; P_t is the phage assay at time t minutes; D is the final dilution of antiserum; and k minutes^{-1} is the first order inactivation constant. Certain conditions necessary to obtain k have previously been described (6). Because in previous studies the neutralizing activity of several sera containing 19S antibody fell after storage at -10°C for 6 months, all antibody titrations were performed within several weeks after obtaining the serum samples. Titrations were usually done at the same time on a series of bleedings from one animal and its control. These sera usually defined the kinetics of a particular antibody response, *e.g.*, the primary 19S response.

Double diffusion in agar was carried out by the method of Preer (12) using as antigen ϕX (A) at a concentration of 2.4×10^{11}/ml. A sonicated preparation of *E. coli* C (10 kv for 15 minutes of a culture containing 5×10^9 organisms/ml) was used to detect precipitating antibody against bacterial antigens. The tubes were kept on their sides at 4°C and observed for 4 weeks.

Physicochemical Characterization of Antibody.—Density gradient ultracentrifugation was kindly performed for us by Dr. E. Franklin. 0.2 to 0.25 ml whole serum was layered over a gradient formed from 21, 14, and 7 per cent saline in a Spinco model L ultracentrifuge with an SW 39 swinging-bucket rotor (13) at 22,000 RPM for 15 to 16 hours. The fractions were collected through a small perforation placed at the bottom of the centrifuge tube. Protein concentrations were determined by the Folin-Ciocalteu method (14). Samples were then pooled to obtain a rapidly sedimenting fraction rich in 19S γ-globulin and essentially free of 7S γ-globulin molecules, and a second fraction containing the bulk of 7S γ-globulin and only small amounts of 19S γ-globulin. An intermediate fraction containing a mixture of the two types of molecules was not used. The fractions were extensively dialyzed against normal saline prior to assay for antibody activity.

Antibody was also treated with 2-mercaptoethanol (2-ME). A 1:6.7 or more dilution of serum in a final concentration of 0.1 M 2-ME was incubated for 30 minutes at 37°C. Serum diluted less than 1:6.7 usually developed macroscopic precipitation. Titrations of antibody were performed without the removal of 2-ME.

X-Irradiation.—400 r whole body x-irradiation was administered to 2 kg rabbits by a 220 kv Picker x-ray machine (Picker X-Ray Corp., Cleveland). The rabbits received half their irradiation from each side at a distance of 38 cm from the tube. With the machine operating at 220 kv and 20 ma, and using a filter of 0.5 mm of copper and 1 mm of aluminum, 51 r per minute were delivered as a midphantom dose as measured by a Victoreen ionization meter (The Victoreen Instrument Co., New York) on a revolving platform.

RESULTS

Sedimentation Properties and 2-Mercaptoethanol (2-ME) Susceptibility.— Previous studies of gamma globulin molecules formed in humans (15), rabbits (4), and chickens (9) revealed that rapidly sedimenting molecules (19S) are depolymerized by 0.1 M 2-ME with complete loss of antibody activity. In contrast, slowly sedimenting (7S) molecules do not lose antibody activity after this treatment.

To test for a similar correlation between sedimentability and susceptibility to 2-ME treatment with guinea pig gamma globulin, two guinea pigs were immunized with 10^{10} ϕX. One animal was bled at 9 days, the second at 7 and 17. A third guinea pig was injected twice with 10^{11} ϕX 4 weeks apart and bled 4 days after the second challenge. Using these 4 sera, antibody determinations were performed on untreated and 2-ME-treated serum, and on fractions obtained by ultracentrifugation in a saline gradient.

TABLE I

Sedimentability and 2-Mercaptoethanol (2-ME) Susceptibility of Guinea Pig Antibody to ϕX

Days after Immunization*	k of serum			
	Untreated	After treatment with 2-ME‡	Rapidly sedimenting	Slowly sedimenting
7	8.7	1.7	12	2.5
9	12	0.35	15	5.5
17	63	96	5.9	89
32§	1100	1100	39	1500

* Injected intravenously with 10^{10} ϕX.

‡ 0.1 M 2-ME for 30 minutes at 37°C.

§ Injected twice intravenously with 10^{11} ϕX 4 weeks apart and bled 4 days after second challenge.

As shown in Table I, rapidly sedimenting molecules were susceptible to inactivation by 2-ME; slowly sedimenting molecules were usually not. In the 17 day serum, k was increased when 2-ME was added. This apparent increase in neutralizing capacity of slowly sedimenting antibody after 2-ME treatment occurred frequently throughout these experiments.

Thus, the sedimentation properties of guinea pig antibody molecules are correlated with their susceptibility to 2-ME: *i.e.*, rapidly sedimenting in contrast to slowly sedimenting antibody is inactivated by 2-ME.

In the remainder of this paper, 2-ME susceptibility will be used to classify antibody molecules as either rapidly or slowly sedimenting. For simplicity, these two classes will be called 19S and 7S, since, by analogy with other mammalian gamma globulins, these are the probable sedimentation constants. It

should be kept in mind, however, that the precise Svedbergs of these molecules have not been determined, and that other types of gamma globulin may be included in these two groups. For example, if a class of intermediate sedimenting antibody, recently described by Rockey and Kunkel in humans (16), also occurs as an antibody to ϕX in guinea pigs, such antibodies would be included in the 19S group.

Half-Life of 19S Homologous Antibody.—Previous studies of anti-ϕX formation in guinea pigs demonstrated that between 1 and 2 weeks after immunization, there was a change in serum antibody molecules from 19S to 7S (6). This observation suggested that synthesis of 19S had stopped, and that 19S molecules have a relatively short half-life.

To investigate the fate of 19S molecules, 3 guinea pigs were injected with 10^{11} ϕX and bled at 7 days. Their pooled serum had a k value of 8.0 and all antibody activity was lost after 2-ME treatment. A pool of 7S anti-ϕX with a k of 6.2, was obtained from the same animals 3 to 4 weeks after immunization. 0.5 ml of 19S anti-ϕX was injected intravenously into each of four 400 gm guinea pigs and 0.85 ml of the 7S anti-ϕX into each of 3 guinea pigs. The animals were bled at intervals, and the k value of the sera was determined.

The mean biological half-life of 19S anti-ϕX was 25.8 hours (range 23 to 31); an equilibration between intra- and extravascular compartments was observed in 2 of 4 animals. In agreement with others (17), the mean half-life of 7S antibody activity was 5.5 days (range 5 to 6) following an initial equilibration in all. Fig. 1 shows the results for one representative animal of each group.

Taliaferro and Talmage (18) have shown that large antibody molecules in the rabbit with hemolytic activity to sheep red cells have a half-life of approximately 3 days, in contrast to small molecules of similar specificity which have a half-life of approximately 5.5 days.

Kinetics of the Primary 19S Antibody Response.—Previous studies of the kinetics of 19S anti-ϕX formation revealed an initial exponential increase in serum antibody with a similar relative rate[1] of antibody formation over a 10^4 dose range of phage (6). 10^9 ϕX of a different preparation (A), usually stimulated a 19S response without a detectable 7S response.[2] It was thus possible to

[1] The term "relative rate of antibody formation" has been used for the rate constant k of the exponential rise of serum antibody (in the form e^{kt}) and represents the slope of that curve plotted on semilogarithmic paper. The time required for doubling the serum antibody level is one measure of the relative rate.

[2] The antigenicity of different preparations of bacteriophage ϕX 174 may vary considerably. For example, a preparation of ϕX previously described (6) was consistently capable of immunizing guinea pigs with as little as 10^4 plaque-forming particles. In contrast, the two preparations of ϕX used in this study required approximately 10^8 plaque-forming particles to immunize detectably. The titers of the freshly prepared lysates before purification in these two groups were comparable as was the low endotoxin content of the purified material suggesting that the immunogenic difference observed is not primarily due either to differences in total phage concentration or endotoxin content.

study the decline in 19S antibody levels without interference by 7S formation. This decline could also be analyzed, but less accurately, in the presence of 7S antibody formation by performing titrations on both untreated and 2-ME–treated antiserum and subtracting k values. The kinetics of anti-ϕX formation

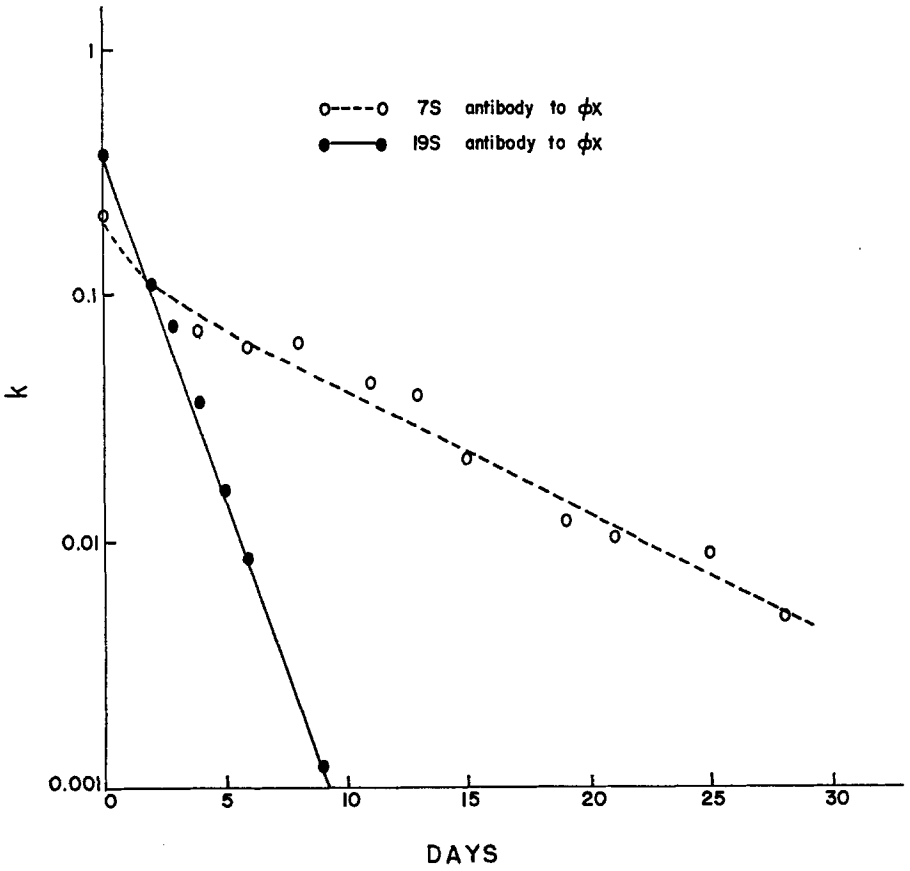

FIG. 1. Elimination of passively administered homologous 19S and 7S anti-ϕX from the circulation of the guinea pig. The elimination curves of two representative animals, one of which received 19S and the other 7S are shown.

was studied in groups of 2 to 5 animals injected with varying doses of ϕX: 10^9, 10^9 with endotoxin, and 10^{11}.

Fig. 2 shows the kinetics in 4 representative animals. Three animals immunized with either 10^{11}, 10^9 (A), or 10^9 (A) with endotoxin had a similar relative rate of antibody formation; *i.e.*, the time for doubling the serum antibody levels was approximately 7 hours. Of 12 animals receiving 10^9 or 10^{11} ϕX,

all but three had a doubling time between 6 to 8.5 hours. These three animals, however, each of which received 10^9 ϕX (A) (one is shown in Fig. 2), had longer doubling times: 10, 15, and 16 hours. These findings indicate that the relative rate of primary antibody formation is dose-independent above a

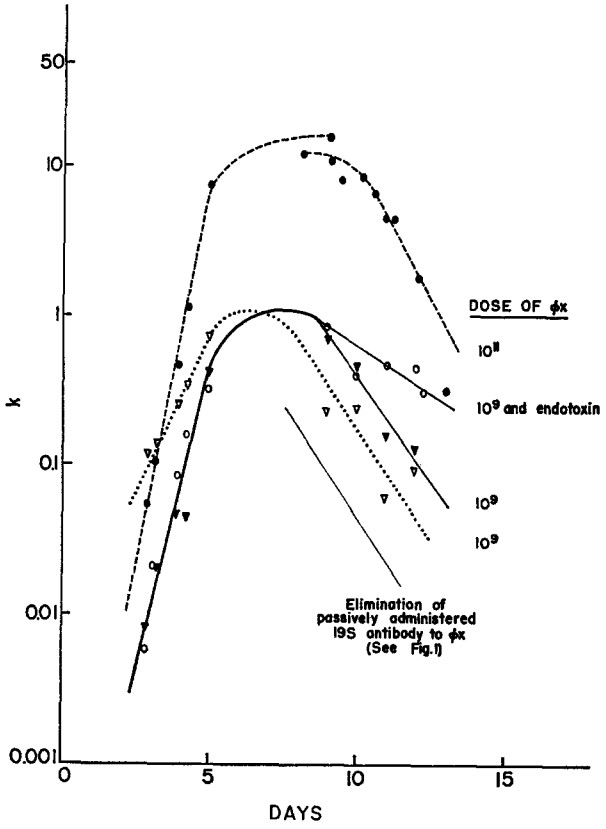

FIG. 2. The primary 19S antibody response to ϕX in the guinea pig. The responses of representative animals injected with 10^9, 10^9 and endotoxin, or 10^{11} ϕX (A) are shown.

"threshold" dose of antigen. Below this dose, lower relative rates of antibody formation can be obtained. Since serum antibody cannot be measured until ϕX is eliminated from the circulation (3 days after immunization), it is possible that a greater relative rate of antibody synthesis preceded the observed relative rate; such a sequence was described by Taliaferro and Taliaferro for hemolysin production in rabbits (19, 20).

It can also be seen that within 10 days after injection of 10^9 or 10^{11} ϕX, 19S formation virtually ceased in these animals as shown by the similarity in

the rates of decline of their serum antibody and of passively administered 19S antibody in normal animals.

Johnson *et al.* (21) have shown that endotoxin administered to rabbits at the time of immunization nonspecifically increased the antibody titer at 1 week; this effect was not obtained in guinea pigs. In the present study, the injection of endotoxin with 10^9 ϕX, did not significantly affect either the relative rate of antibody formation or the peak titer, but 19S antibody levels remained higher for a longer period of time in all of 3 guinea pigs tested. This finding can be interpreted as a prolongation of 19S antibody synthesis by administration of endotoxin, since passively administered 19S antibody had the expected half-life of 24 hours in a guinea pig injected with endotoxin 10 days previously.

Kinetics of the Primary 7S Antibody Response.—The kinetics of 7S antibody formation was determined by measuring antibody in the presence of 0.1 M 2-ME. Groups of 2 to 6 animals were injected with 10^9–10^{11} ϕX and bled at intervals. Some of these animals had been bled previously for determination of the kinetics of 19S antibody formation.

Fig. 3 shows the results in 3 representative animals injected with either 10^9 (B), 10^{10}, or 10^{11} ϕX. In each, serum antibody was detected on day 7 or 8, and antibody levels rose exponentially for 4 to 5 days. As can be seen, the relative rate of antibody formation increased as the dose of phage for immunization was increased. The mean doubling times in hours for 2 animals receiving 10^9 ϕX (B) was 22 (19 and 24); for 4 animals receiving 5×10^9 (A), 24 (range 17 to 31) (not shown in Fig. 3); for 5 animals receiving 10^{10}, 17 (range 15 to 19); and for 5 receiving 10^{11}, 14 (range 11 to 17). In contrast to the primary 19S response, there was no evidence that a maximal relative rate of 7S formation had been obtained.

Following this exponential phase, synthesis continued at a slower relative rate for at least several weeks as indicated by rising or maintained serum antibody levels in all guinea pigs tested. Three animals were primarily immunized with 2×10^{10} ϕX (distributed into all 4 footpads) and bled 8 and 10 months later. Serum k was 31, 71, and 79 at 8 months, and was essentially unchanged at 10 months, indicating that during this period of time substantial amounts of 7S had been formed.

Kinetics of the Secondary Antibody Response.—It was previously shown that the secondary anti-ϕX response has a relative rate approximately equal to, or occasionally less than, the primary response (6). Groups of 2 guinea pigs previously immunized with either 10^9 (B) or 10^{11} ϕX were challenged 4 weeks later with either 10^9 (A), 10^{10}, or 10^{11}. Serum was obtained at intervals after secondary challenge.

Fig. 4 shows representative animals of the 4 groups studied: A, B, and C were injected 4 weeks previously with 10^{11} ϕX and then injected for the second

time with either 10^{11}, 10^{10}, or 10^{9} ϕX, respectively. As can be seen, the relative rate of antibody formation decreased as the dose of antigen for secondary challenge was decreased.

The doubling times for the two animals in the 10^{11} challenged group were

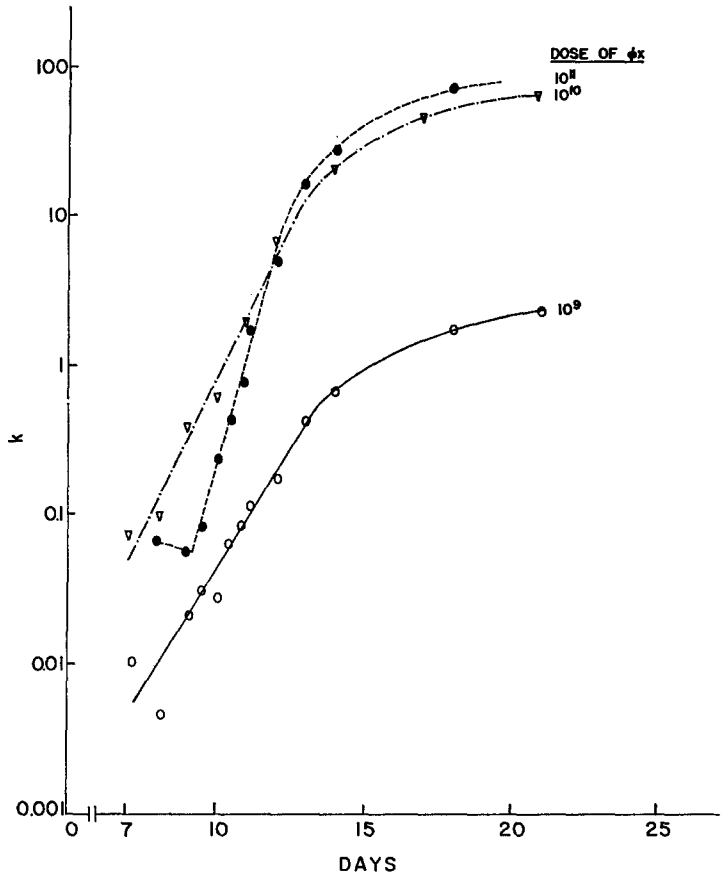

FIG. 3. The primary 7S antibody response to ϕX in the guinea pig. The responses of representative animals injected with either 10^{9} (B), 10^{10}, or 10^{11} ϕX are shown.

6.6 and 9.0 hours; in the 10^{10} group: 23 and 22 hours; and in the 10^{9} group: 34 and 72 hours. Group D previously immunized with 10^{9} (B) phage and challenged with 10^{10} ϕX had doubling times of 8.0 and 10 hours; *i.e.*, in this respect, group D behaved like group A. Presumably, the small dose used for the first immunizing injection resulted in a smaller population of sensitized cells; therefore, the second dose of phage was not distributed to as many cells as in the 10^{11}-primed group.

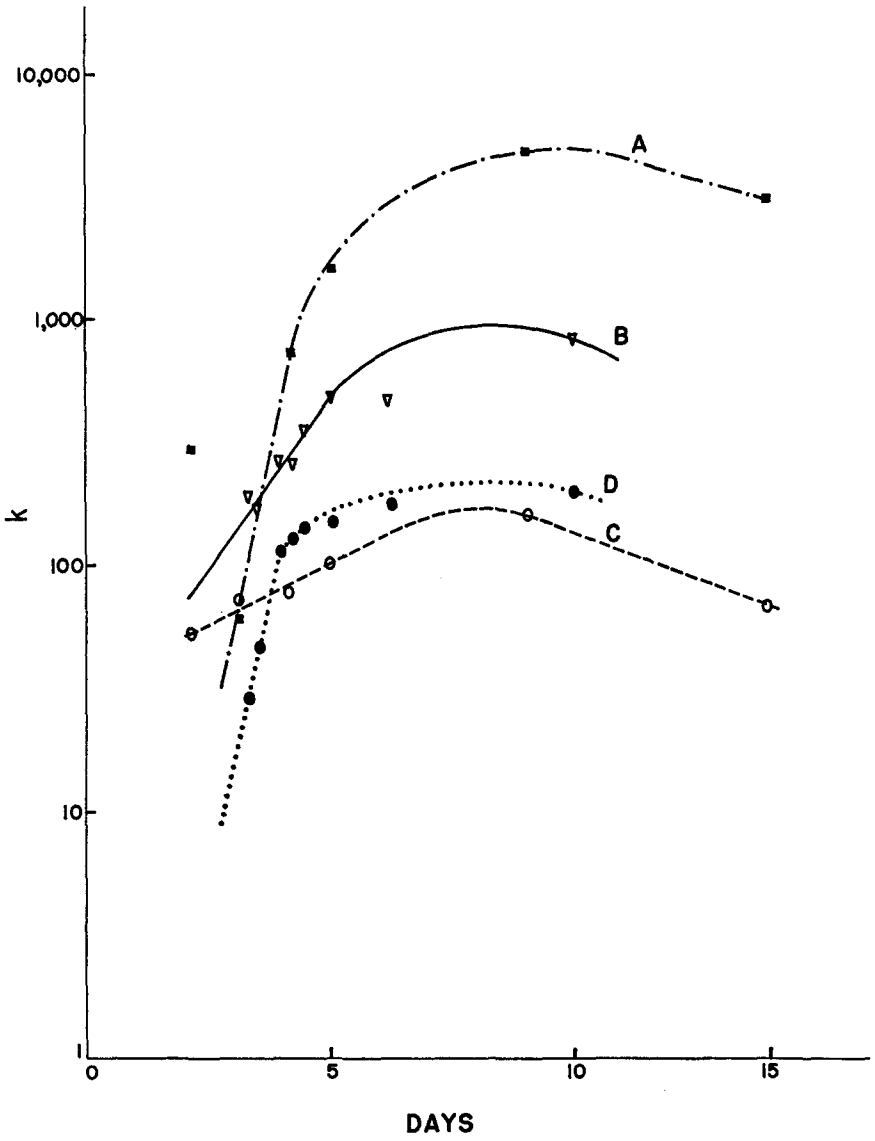

FIG. 4. The secondary 7S antibody response to ϕX in the guinea pig. *A*, *B*, and *C* are the responses of representative animals that received 10^{11} ϕX 1 month previously and were reinjected with 10^{11}, 10^{10}, and 10^{9} ϕX, respectively. *D* is the response of a representative animal injected one month previously with 10^{9} (B) ϕX, and reinjected with 10^{10} ϕX.

These observations suggest that in the secondary response the dose of antigen *in relation* to the size of the sensitized cell population determines the relative rate of antibody formation.

If the more rapid relative rates obtained in these studies are corrected for the contribution of 7S antibody from the primary response, relative rates are obtained which fall in the range of the maximal primary 19S rates. It is possible, of course, that even more rapid 7S relative rates can be obtained since these experiments did not indicate that a maximal rate was achieved. Moreover, since no information was obtained on the kinetics of secondary antibody formation until 2 to 3 days after challenge, the initial relative rates of synthesis may have been higher than the observed rates.

Effect of Whole Body X-Irradiation on 19 and 7S Antibody Response in Rabbits.—The studies above of the primary 7S response indicated that the exponential phase of anti-ϕX formation was not detected until approximately 1 week after immunization. It was possible, therefore, that cells destined to make 7S antibody had not yet been stimulated by antigen; and, in accord with current hypothesis (22), might have an antibody synthesizing mechanism that was relatively susceptible to ionizing radiation.

Accordingly, 8 rabbits were immunized with 10^{10} ϕX, 24 hours later received 400 r whole body x-irradiation, and after a further 24 hours, 4 were injected with 10^9 T_2. A second group received in order: T_2, x-irradiation, and ϕX; a third group injected with ϕX was not x-irradiated.

The purpose of the T_2 immunization was to exclude the possibility of a non-specific protective effect on lymphoid tissue from injection of bacteriophage as previously described for carbon particles (23). Sera were obtained at weekly intervals from all animals.

Table II shows the results of the antibody determinations. At 1 week, 19S anti-ϕX formation was similar in unirradiated controls and in animals receiving ϕX prior to irradiation. However, at 2 to 4 weeks, 5 of 8 animals in the latter group were continuing to produce large amounts of 19S. By comparison with the unirradiated controls, the onset of the primary 7S response did not appear to be delayed in the group receiving ϕX before irradiation; indeed, larger amounts of 7S were formed. The group irradiated before injection of ϕX developed only trace amounts of anti-ϕX, as expected.

Thus, x-irradiation after immunization did not prevent the primary 7S response. Taliaferro and Taliaferro (24) and Dixon (25) have shown that x-irradiation administered to rabbits after injection of antigen increases the peak titer of the antibody response. The present studies indicate that this effect results from both prolongation of 19S synthesis and formation of increased amounts of 7S.

Effect of a Second Injection of ϕX, 5 or 9 Days after Primary Immunization.—It was possible that a second administration of antigen would prolong 19S

synthesis and, in addition, might affect the time of onset and the kinetics of 7S formation.

Seven guinea pigs were injected with 10^{10} ϕX and 5 days later 4 were rechallenged with 10^{11} ϕX. Another six animals were injected with 5×10^9 ϕX, and 9 days later, 4 were rechallenged with 10^{11} ϕX and 2 with 10^{10} T_2. The kinetics of the 19S and 7S primary antibody responses were determined.

TABLE II

Effect of 400 r Whole Body X-Irradiation on the 19S and 7S Antibody Response in Rabbits

Immunization*		Serum anti-ϕX(k)		
24 hrs. before x-ray	24 hrs. after x-ray	1 wk.‡	2 wks.	3 to 4 wks.
Unirradiated ϕX		30/0§	0/59	0/26
		58/0	1.5/50	0/15
		21/0	0/59	—
		26/0	0/70	—
ϕX	T_2	13/0	0/43	—
		25/0	0/160	—
		56/0	97/30	0/350
		18/0	19/42	0/100
		29/0	0/100	—
		16/0	37/230	—
		47/0	39/42	0.7/66
		36/4.0	18/20	24/52
T_2	ϕX	0.04/0.5	0/6.0	—
		0.10/0	1.4/0.04	—
		0.26/0.01	3.5/0.08	1.8/1.3
		0.10/0.01	1.3/0.15	—

— = not done.

* Injected intravenously with 10^{10} ϕX and/or 10^9 T_2.

‡ One week after x-irradiation or in the unirradiated group, after ϕX immunization.

§ Numerator represents 19S; 0 means k less than 15 per cent of total k present; denominator represents 7S; 0 means k less than 10 per cent of total k present.

Fig. 5 shows 4 representative animals of the two experiments. The second injection of ϕX on days 5 or 9 stimulated further 19S formation, although in the latter group 19S formation had virtually ceased, as indicated by the declining slope of 19S in the control group. In the controls, anti-T_2 k's 5 days after T_2 injection on day 9 were less than 1, the expected values. Thus, the prompt rise of 19S levels in the 9 day group indicated that the same population of cells that had previously produced 19S was again synthesizing antibody; otherwise a delay of 5 days or longer would be expected before a new popula-

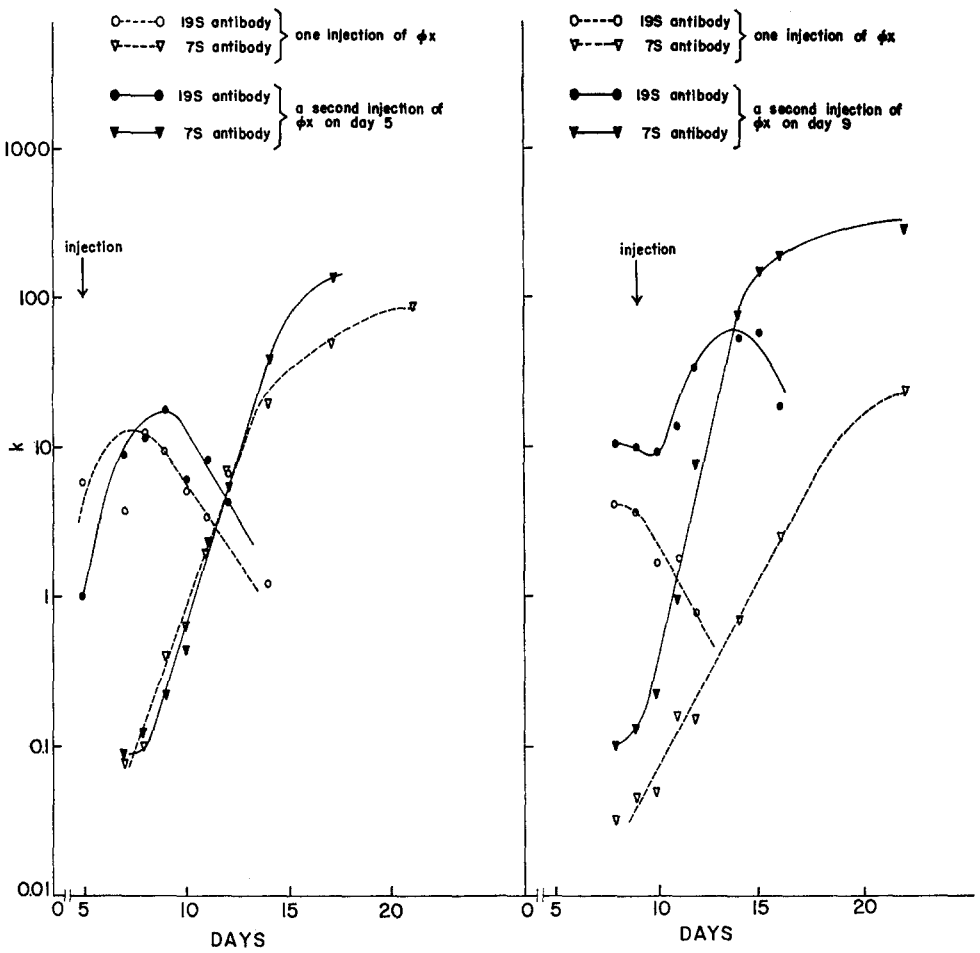

FIG. 5. The effect of a second injection of ϕX on day 5 or 9 on the primary 19S and 7S anti-ϕX responses in the guinea pig. The responses of representative animals are shown. On the left, both animals were injected with 10^{10} ϕX, and one was reinjected with 10^{11} ϕX on day 5. On the right, both animals received 5×10^{9} ϕX; one was reinjected on day 9 with 10^{11} ϕX, and the other with 10^{10} T_2.

tion of cells had produced enough antibody to affect, noticeably, existing antibody levels. Two alternative possibilities had been excluded by the T_2 control group: (*a*) a *non-specific* effect of the additional ϕX on anti-ϕX formation, and (*b*) a *non-specific* change in the lymphoid organs of immunized animals that would allow previously uncommitted cells to give an accelerated anti-ϕX response to the second injection of ϕX. In this experiment, therefore, the cessation of 19S synthesis was due to antigen depletion.

This experiment also showed that the prolongation of 19S synthesis by injection of ϕX on either day 5 or 9 did not noticeably alter the time of onset of 7S formation. As expected, however, the relative rate of 7S formation was increased by the additional ϕX, but was still less than maximal relative rates obtained in the primary 19S response.

The Relationship of the Primary 19S and Primary 7S Responses to Preparation for a Secondary Response (Immunological Memory).—The inability to detect substantial amounts of 19S during a secondary antibody response[3] suggested that 19S formation might not lead to immunological memory. It was possible to test this hypothesis since injection of 10^9 ϕX (A) resulted in a 19S response without detectable 7S.

Four guinea pigs were injected with 10^9 ϕX(A), and 4 weeks later 2 were reinjected with 10^9 (A) and 2 with 10^{11} ϕX. One animal in each group was reinjected for the third time 4 weeks later with 10^9 ϕX (A). Sera were obtained at frequent intervals after the second and third injections.

Fig. 6 shows the results of separate antibody responses to three injections in two representative animals. The first 10^9 (A) response is the expected one (see Fig. 2); the second and third responses represent observed values. It was not practical to attempt three series of bleedings in the same animal. As can be seen, the second and third injections of 10^9 ϕX (A) given at 1 month intervals resulted in 19S responses closely resembling the response to the first injection, (peak $k < 5$). The other animal in that group died shortly after the third injection, but its response to the second injection also resembled the primary 19S response. Fig. 6 also shows a representative response of one of two animals injected for the second time with 10^{11} ϕX. This second 19S response closely resembled the primary one to 10^{11} ϕX (Fig. 2), and in addition was followed by the expected primary 7S response (as in Fig. 3). Rechallenge after another 4 weeks with 10^9 ϕX (A) resulted in a secondary 7S response[4] (as in Fig. 4). Eleven other animals injected with 10^9 (B) or 10^{11} ϕX all developed a primary 7S response and 7S immunological memory.

The failure of a primary 19S response to lead to immunological memory could be due to a quantitative deficiency in the antibody response. It was therefore necessary to relate the neutralizing capacity (k) to the concentration

[3] When 19S and 7S anti-ϕX were mixed in varying ratios and titered with and without 2-ME, 19S was consistently detected at concentrations of 15 per cent or more of the total serum k.

[4] Our studies have revealed four differences between the secondary compared to the primary 7S response to the same dose of antigen. These are: (1) "latent period" shorter; (2) greater *absolute* rate of antibody formation; (3) higher peak titer; and (4) peak titer reached earlier.

It is not known if these criteria will be useful for other antigens or animal species; or whether or not a first and second response to antigen should be compared with reference to the class of γ-globulin.

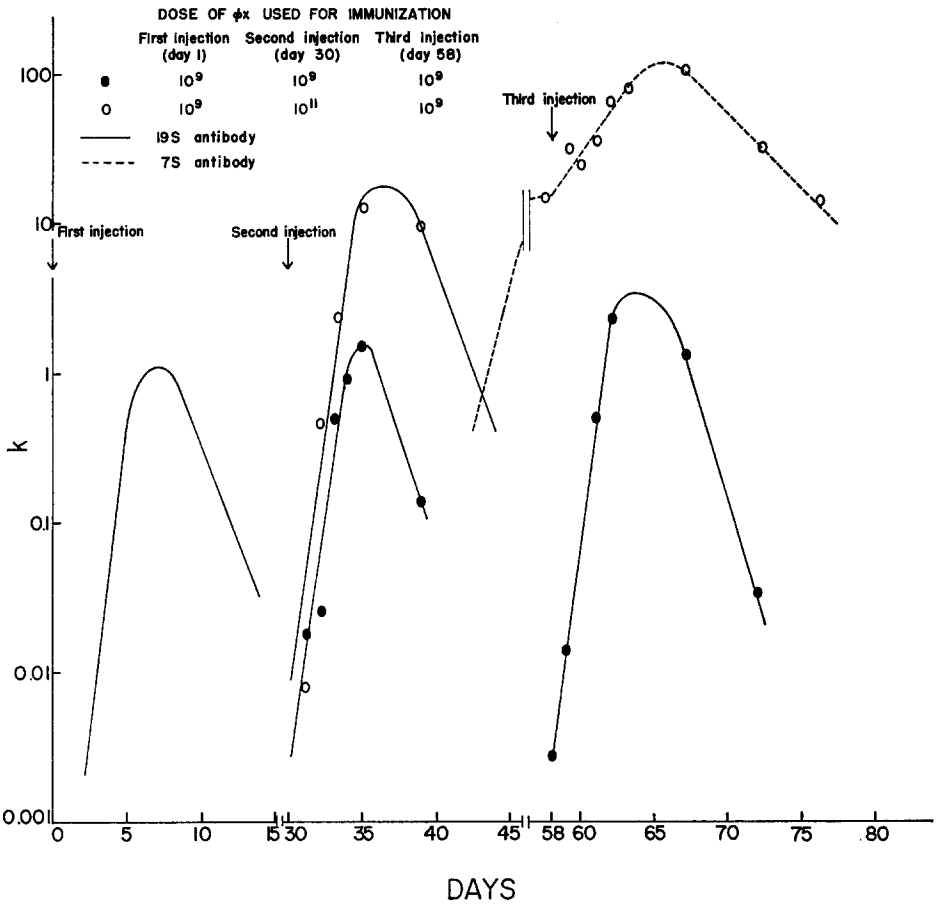

FIG. 6. The relationship of the primary 19S and 7S responses to immunological memory in the guinea pig. The responses of two representative animals are shown: one was injected three times with 10^9 (A) at 1 month intervals; the second was injected with 10^9 (A), 10^{11} and 10^9 (A) also at 1 month intervals. The first antibody response is the expected response of both animals based on the responses of analogously immunized animals; the second and third antibody responses represent actual determinations.

of antibody present. Double Preer agar diffusion was used to determine if precipitating antibody was present, since only small amounts of ϕX are required for this procedure. Using ϕX or a sonicated preparation of *E. coli* C as antigen, the following sera were tested: six 19S sera, obtained from 6 guinea pigs immunized with 10^9 ϕX (A or B), including 2 of the preceding animals; two 7S sera obtained during a primary response; and 2 sera obtained during a secondary 7S response. All 19S anti-ϕX sera with ϕX, but not *E. coli*, as

antigen formed a single line of precipitation close to the antibody layer after 1 to 2 weeks. A 19S serum with a higher k was diluted 1:10 in saline or 0.1 M 2-ME. The presence of 2-ME prevented the development of the precipitation line. The four 7S sera each formed a precipitation line that was closer to the antigen layer. There was no indication from serial dilutions that antibody formed during the 7S primary response was grossly less efficient in neutralization than 19S.

TABLE III

The Relationship of the Primary 19S and 7S Antibody Responses to the Preparation for a Secondary Antibody Response in Guinea Pigs

Dose of ϕX used for primary and secondary immunization*	No. of animals	Antibody response after first immunization‡		Antibody response 1 wk. after second immunization‡	
		19S at 1 wk.	7S at 3 to 4 wks.	19S	7S
3×10^8	4	0.20 (0.043 to 0.48)	0	0.17 (0.075 to 0.33)	0
10^9	4	0.97 (0.23 to 2.2)	0	0.70 (0.53 to 0.96)	0
3×10^9 (*a*)	1	0.53	0	1.4	0
(*b*)	1	0.41	0	2.1	1.7
(*c*)	2	1.8 (0.33 and 3.3)	0.59 (0.20 and 0.98)	0	11 (8.7 and 14)
6×10^9	2	0.63 (0.50 and 0.77)	0.59 (0.55 and 0.63)	0	21 (14 and 28)
10^9 and endotoxin§ (*a*)	1	0.46	0	0.99	2.5
(*b*)	1	0.81	0.05	1.6	4.1

* Dilutions of ϕX (A) diluted in saline injected intravenously. Same dose of phage was used for the first and second immunization.

‡ Mean k presented. Range of values are shown in parentheses. For 7S, 0 = k less than 0.01; for 19S, 0 = k less than 15 per cent of total k.

§ Animals received 10 μg of endotoxin in saline intraperitoneally at time of primary immunization.

To confirm and extend the results of the first experiment, an additional experiment was performed in which groups of 4 guinea pigs were immunized twice, 1 month apart with 3×10^8, 10^9, 3×10^9, or 6×10^9 ϕX (A). Serum was obtained weekly for 4 weeks after the first injection, and 1 week after the second.

Table III summarizes the results of this experiment. In the 3×10^8 and 10^9 groups, and in one animal in the 3×10^9 group, the first and second responses were remarkably similar, both consisting of 19S without detectable 7S. Al-

though 5 of 9 animals had a higher peak titer during the second 19S response compared to the first, the mean k's of the two responses were not significantly different: first response, 0.59 (0.043 to 2.2); second response, 0.54 (0.075 to 1.4). In those animals receiving 6×10^9 and in two receiving $3 \times 10^9 \phi X$, a primary 19S response was followed by a primary 7S response and 7S immunological memory. These results confirm those of the previous experiment.

One group of four guinea pigs that received 3×10^9 ϕX (A) twice, one month apart, showed three different types of antibody responses. Two guinea pigs developed both a 19S and 7S response, and two developed only 19S. Of the latter group, one animal in contrast to the others in this experiment, showed 7S immunological memory (Table III). This animal also developed a detectable 19S response during the secondary 7S response. One of two other guinea pigs shown in Table III that received endotoxin with their first injection behaved in an analogous fashion. In these two animals which developed 7S immunological memory without a detectable primary 7S response, undetected amounts of 7S may have been present, or perhaps the threshold of antigen for proliferation of 7S cells is less than that for detectable 7S synthesis, as previously suggested in specific-precipitate–immunized guinea pigs (26).

DISCUSSION

The studies reported here extend previous observations on the kinetics of the primary and secondary antibody responses to bacteriophage ϕX 174 in the guinea pig (6).

It has been shown that 19S antibody (19S) formation is short-lived; it virtually ceases within 10 days after injection of 10^{11} ϕX particles (4 to 6 days after completion of the exponential phase of antibody synthesis) and 19S serum levels then decline with a half-life of approximately 24 hours. The duration of 19S synthesis can be prolonged for several days, however, by a second injection of ϕX given 5 or 9 days after the first (Fig. 4). If the second injection is given on day 9, serum antibody levels begin to rise within 24 hours, indicating synthesis by the same population of cells which previously produced 19S. If a new population of cells had responded, a delay of 5 to 7 days would have been expected before antibody production was sufficient to increase noticeably the existing high serum antibody level. In these experiments, therefore, cessation of 19S synthesis was due primarily to antigen lack. Stated from another viewpoint, there is at least one immune response, primary 19S formation, in which the presence of antigen appears to be necessary for continued synthesis of antibody. Other physiological factors may possibly place an upper limit on the prolongation of 19S formation by antigen. For example, the duration of 19S synthesis was also increased by either injection of endotoxin with antigen or, more strikingly, by 400 r whole body x-irradiation 24 hours after antigen injection. Although both experimental procedures

may affect antigen catabolism, irradiation may also have been effective by its removal of neighboring non-antibody producing cells and/or the local increase of nucleotides (27).

At approximately the time that the relative rate (see footnote 1) of 19S synthesis declines, 7S antibody (7S) formation can first be definitely detected. The cause for the delay in 7S production is unknown. This delay is probably not due to failure of antigen to make contact with a "prepared" cell, since antigen circulates for at least 72 hours, and it cannot be attributed to different efficiencies in neutralization of ϕX by 19S and 7S according to agar diffusion studies. Presumably, the delay in 7S synthesis represents the time for completion of other events, possibly 19S synthesis. In marked contrast to 19S synthesis, formation of substantial amounts of 7S have been demonstrated 10 months after a single injection of phage-in-saline, suggesting that 7S synthesis may continue for the lifetime of a guinea pig. Thus, if persistence of antigen is essential for continuation of synthesis of all classes of antibody, it can be concluded that the 19S system is rapidly depleted of antigen, the 7S system is not.

Further experiments on the kinetics of anti-ϕX formation indicate that antigen may play a role in the regulation of the relative rate of antibody synthesis. Below a critical level of antigen, the relative rate of antibody formation is antigen-dependent. Above this level, at least in the primary 19S response, the rate appears to be antigen-independent with a maximal rate of 6 hours for doubling the serum *k*. This maximal rate cannot be explained solely on the basis of proliferation of a population of antibody-producing cells, since previous studies of lymphoid and other mammalian cells suggest 10 to 12 hours as a maximal rate for mitosis (28–30); nor can this rate be attributed to the continual recruitment of immunologically virgin cells for antibody formation, since similar rapid rates were obtained in the secondary response in which antibody formation by previously uncommitted cells would not be noticeable. These findings have led to the conclusion that during the exponential phase, the *absolute* rate of antibody formation per cell is increasing; and, as previously suggested (6) at a constant rate (*i.e.*, the *relative* rate is constant). To explain the exponential character of this increase, a differentiation process within the antibody producing cell must be invoked, for example, replication of ribosomes.

Although maximal rates were obtained in the primary 19S response, and probably in the secondary 7S response, there was no evidence that even two injections of ϕX, 5 or 9 days apart, stimulated maximal kinetics in the primary 7S response. This finding was unexpected, since factors operating against maximal rates of antibody synthesis in the primary 7S response (excess serum antibody, larger cell population than that engaged in primary 19S synthesis, etc.) would be expected to be more active in the secondary 7S response. In addition, the amount of antigen necessary to stimulate a detectable antibody

response was larger for the primary 7S response than for either the primary 19S response, or the secondary 7S response. These operational differences suggest differences in the mechanisms responsible for these three antibody responses but do not indicate whether a single or different cell type is responsible.

Perhaps the most provocative finding to emerge from these studies is the relationship of the 19S- and 7S-producing mechanisms to preparation for a secondary 19S or 7S response (immunological memory). The short-lived nature of 19S synthesis and the failure to detect significant amounts of 19S during a secondary 7S response raises the possibility that the 19S mechanism does not lead to immunological memory. Strong evidence for this possibility was the finding that small doses of ϕX (3×10^8 and 10^9 (A)) which stimulated 19S formation without 7S in guinea pigs did not lead to immunological memory. Of 12 animals reimmunized 1 month later with the initial small dose of ϕX, all showed a second 19S response which closely resembled the primary one, and all failed to develop detectable 7S at 1 week. Indeed, in one guinea pig that received a third injection of 10^9 ϕX (A) after an additional 1 month interval, there was a third 19S response without detectable 7S (see Fig. 6). Although there was no significant difference between the mean peak *k*'s of both 19S responses in ten animals whose sera were titered at the same time, five of them had a higher peak *k* during the secondary compared to the primary 19S response. In any event, the striking immunological memory displayed by the 19S system after reimmunization on day 9 (see Fig. 4) did not persist for 1 month. In contrast to the above group, all 15 animals that developed a primary 7S response, including those with barely detectable 7S, developed 7S immunological memory that was clearly demonstrated at one month (see Table III and footnote 4).

It is unlikely that the failure of the 19S response to persist and lead to immunological memory can be explained by a quantitative deficiency in the 19S response, since in this group that did not develop 7S immunological memory: (*a*) 19S precipitating antibody to ϕX was demonstrated by double Preer agar diffusion in all 5 sera tested, indicating that a minimum of several micrograms of antibody protein/ml was present; (*b*) the primary 19S response of two of these animals was greater than the primary 19S response of two guinea pigs that *did* develop 7S immunological memory. These findings suggest that, in contrast to 7S, the 19S mechanism does not develop a persisting immunological memory.

At least four possibilities could account for the failure of the cells responsible for 19S formation to continue antibody synthesis and develop persisting immunological memory: (*a*) death of cells; (*b*) repression of 19S mechanism; (*c*) transfer of immunological information from cells making 19S (from a clonal selection viewpoint, the small lymphocyte (31)) to cells that can then form 7S antibody (the large lymphocyte (30)) by a transformation process; (*d*) antigen depletion, if antigen is essential for immunological memory.

Our studies support the latter possibility and suggest that the level and retention of antigen by immunologically competent cells may function as a major controlling mechanism for regulating several important immunological functions.

SUMMARY

Injection of a sufficient dose of bacteriophage ϕX 174 into guinea pigs results in the formation of rapidly sedimenting antibody molecules (19S), and later, slowly sedimenting molecules (7S). Above a threshold dose of antigen, the relative rate of 19S formation is maximal and dose-independent; below this dose, slower relative rates are obtained. The time for doubling the serum 19S level is as short as 6 to 8 hours, suggesting that the absolute rate of antibody formation per cell is increasing in addition to proliferation of antibody-producing cells. Synthesis of 19S after injection of 10^{10} ϕX virtually ceases at 10 days after which 19S antibody activity disappears from the circulation with a half-life of approximately 24 hours. A second injection of ϕX on day 5 or 9 prolongs 19S synthesis, indicating that antigen not only can regulate the relative rate, but also is essential for continued synthesis of 19S. 19S synthesis is also prolonged in guinea pigs by injection of ϕX with endotoxin or by 400 r whole body x-irradiation 24 hours after injection of phage into rabbits.

The primary 7S response is not detected until approximately 1 week after immunization and relative rates are antigen-dependent. Primary 7S synthesis can continue for many months and leads to preparation for a secondary antibody response (immunological memory) during which only 7S is detected. In contrast, in animals that form precipitating 19S without detectable 7S, a second injection of phage 1 month later results in a second 19S response which closely resembles the first. These findings have led to the suggestion that formation of 19S does not lead to persisting immunological memory.

The authors wish to acknowledge the invaluable technical assistance of Mr. Yuen H. Chinn.

BIBLIOGRAPHY

1. Lo Spalluto, J., Miller, W., Jr., Dorward, B., and Fink, C. W., The formation of macroglobulin antibodies. I. Studies on adult humans, *J. Clin. Inv.*, 1962, **41**, 1415.
2. Fink, C. W., Miller, W., Jr., Dorward, B., and Lo Spalluto, J., The formation of macroglobulin antibodies. II. Studies on neonatal infants and older children, *J. Clin. Inv.*, 1962, **41**, 1422.
3. Stelos, P., and Taliaferro, W. H., Comparative study of rabbit hemolysins to various antigens. II. Hemolysins to the Forssman antigen of guinea pig kidney, human type A red cells, and sheep red cells, *J. Infect. Dis.*, 1959, **104**, 105.
4. Bauer, D. C., and Stavitsky, A. B., On the different molecular forms of antibody synthesized by rabbits during the early response to a single injection of protein and cellular antigens, *Proc. Nat. Acad. Sc.*, 1961, **47**, 1667.

5. Benedict, A. A., Brown, R. J., and Ayengar, R., Physical properties of antibody to bovine serum albumin as demonstrated by hemagglutination, *J. Exp. Med.*, 1962, **115,** 195.
6. Uhr, J. W., Finkelstein, M. S., and Baumann, J. B., Antibody formation. III. The primary and secondary antibody response to bacteriophage ϕX 174 in guinea pigs, *J. Exp. Med.*, 1962, **115,** 655.
7. Uhr, J. W., Dancis, J., Franklin, E. C., Finkelstein, M. S., and Lewis, E. W., The antibody response to bacteriophage ϕX 174 in newborn premature infants, *J. Clin. Inv.*, 1962, **41,** 1509.
8. Silverstein, A. M., Uhr, J. W., Kraner, K. L., and Lukes, R. J., Fetal response to antigenic stimulus. II. Antibody production by the fetal lamb, data to be published.
9. Uhr, J. W., Finkelstein, M. S., and Franklin, E. C., Antibody response to bacteriophage ϕX 174 in non-mammalian vertebrates, *Proc. Soc. Exp. Biol. and Med.*, 1962, **111,** 13.
10. Fraser, D., and Jerrel, E. A., The amino acid composition of T_3 bacteriophage, *J. Biol. Chem.*, 1953, **205,** 291.
11. Adams, M. H., *in* Bacteriophages, New York, Interscience Publishers, 1959.
12. Preer, J. R., A quantitative study of a technique of double diffusion in agar, *J. Immunol.*, 1956, **77,** 52.
13. Edelman, G. M., Kunkel, H. G., and Franklin, E. C., Interaction of the rheumatoid factor with antigen antibody complexes and aggregated gamma globulin, *J. Exp. Med.*, 1958, **108,** 105.
14. Folin, O., and Ciocalteu, V., On tyrosine and trytophane determinations in proteins, *J. Biol. Chem.*, 1927, **73,** 627.
15. Deutsch, H. F., and Morton, J. I., Dissociation of human serum macroglobulins, *Science*, 1957, **125,** 600.
16. Rockey, J. H., and Kunkel, H. G., Unusual sedimentation and sulfhydryl sensitivity of certain isohemagglutinins and skin-sensitizing antibody, *Proc. Soc. Exp. Biol. and Med.*, 1962, **110,** 101.
17. Dixon, F. J., Talmage, D. W., Maurer, P. H., and Deichmiller, M., The half-life of homologous gamma globulin (antibody) in several species, *J. Exp. Med.*, 1952, **96,** 313.
18. Taliaferro, W. H., and Talmage, D. W., Antibodies in the rabbit with different rates of metabolic decay, *J. Infect. Dis.*, 1956, **99,** 21.
19. Taliaferro, W. H., and Taliaferro, L. G., The dynamics of hemolysin formation in intact and splenectomized rabbits, *J. Infect. Dis.*, 1950, **87,** 37.
20. Taliaferro, W. H., and Taliaferro, L. G., The role of the spleen and dynamics of hemolysin production in homologous anamnesis, *J. Infect. Dis.*, 1952, **90,** 205.
21. Johnson, A. G., Gaines, S., and Landy, M., Studies on the O antigen of *Salmonella typhosa*. V. Enhancement of antibody response to protein antigens by purified lipopolysaccharide, *J. Exp. Med.*, 1956, **103,** 225.
22. Dixon, F. J., Talmage, D. W., and Maurer, P. H., Radiosensitive and radioresistant phases in the antibody response, *J. Immunol.*, 1952, **68,** 693.
23. Strauch, D., Stender, H., and Winter, H., Effects of a nonspecific stimulation on

tissue radiosensitivity during the course of antibody production, *J. Immunol.*, 1959, **82,** 298.

24. Taliaferro, W. H., and Taliaferro, L. G., Effect of x-rays on hemolysin formation following various immunization and irradiation procedures, *J. Infect. Dis.*, 1954, **95,** 117.
25. Dixon, F. J., personal communication.
26. Uhr, J. W., and Baumann, J. B., Antibody formation. II. The specific anamnestic antibody response, *J. Exp. Med.*, 1961, **113,** 959.
27. Jaroslow, B. N., and Taliaferro, W. H., The restoration of hemolysin-forming capacity in x-irradiated rabbits by tissue and yeast preparations, *J. Infect. Dis.*, 1956, **98,** 75.
28. The Kinetics of Cellular Proliferation, (F. Stohlman, Jr., editor), New York, Grune and Stratton, 1959.
29. Schooley, J. C., Autoradiographic observations of plasma cell formation, *J. Immunol.*, 1961, **86,** 331.
30. Nossal, G. J., and Mäkelä, O., Autoradiographic studies on the immune response. I. The kinetics of plasma cell proliferation, *J. Exp. Med.*, 1962, **115,** 209.
31. Burnet, F. M., A modification of Jerne's theory of antibody production using the concept of clonal selection, *Australian J. Sc.*, 1957, **20,** 67.