COMPETITION OF 19S AND 7S ANTIGEN RECEPTORS IN THE REGULATION OF THE PRIMARY IMMUNE RESPONSE

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Various theories of antibody formation (1, 2, 3) propose that the primary antigenic stimulus is mediated by preformed specific "natural" antibody molecules.

A large amount of data has now accumulated (4–10) indicating that passively administered specific antibody has a depressive effect on the immune response. Both preparations of 7S antibody and, to a lesser degree, preparations of 19S antibody were found to be depressive. It has been proposed (4) that the antibody acts directly on antigen-sensitive target cells, but the evidence now suggests that antibody inhibits the response by combining with antigenic determinants. Recently, it has been found (11) that when two haptens are present on one carrier molecule, suppression of antibody formation against one determinant does not necessarily suppress antibody formation against the second determinant. It therefore appears that suppression of the response by antibody does not imply a prevention of the antigen from reaching the target area. Possibly, binding of antigenic determinants by antibody reduces the number of free determinants available for stimulation.

Though apparently contradicted by these findings, the theories alluded to in the first paragraph, do not lack experimental support. Segre and Kaeberle (12) found that both specific antibody and "normal" gamma globulin enhance the antibody response of newborn piglets deprived of colostrum. Hemphill et al. (13) terminated tolerance to human serum albumin in mice by injecting antigen-antibody complexes. Möller and Wigzell (7) found a minor enhancement of the number of plaque-forming cells in occasional mice when small amounts of specific antibody were administered prior to an injection of sheep red cells. Williams (14) has demonstrated that the reduced follicular localization of polymerized flagellin observed in X-irradiated and lymphocyte-depleted rats was significantly improved when specific antisera were injected prior to antigen injection. In these studies, the immunoglobulin type of the enhancing antibody was not determined. More recently, Pearlman (15) has reported that whereas both 19S and 7S antibodies are effective inhibitors of antibody formation, both are also capable of enhancement when given in small amounts. In contrast to the depressive effect, enhancement appeared to be nonspecific. Others have reported that, though small amounts of IgG anti-Rh antibody can suppress the response of Rh-negative mothers to the Rh-positive cells of their newborn, relatively small amounts of IgM antibody actually result in enhanced antibody formation (16).

The present studies were initiated in the hope of clarifying some of the questions raised by our studies of the primary immune response of mice to

different doses of red cells.[1,2] Preliminary experiments[1] had indicated that, although small amounts of specific 7S antibody were suppressive when given prior to antigen, equal molar amounts of 19S antibody consistently enhanced the primary immune response. The result of antigenic stimulation after the administration of 7S and 19S antibody appeared to depend on the ratio of these two species of immunoglobulin. We now present additional quantitative data substantiating this claim. An abridged account of some of our findings has been reported (17).

Materials and Methods

Animals.—Most of the data were obtained with inbred NMRI mice aged 6–8 wk. In preliminary experiments, AKR mice of the same age were used. We have observed no difference in the response of these two strains. Because of the wide variation in the responses of individual animals, large numbers of mice must be used to establish an average response with a sufficient degree of confidence. Each experimental point in this paper represents the arithmetic mean of the number of plaque-forming cells (PFC) per spleen obtained in a group of 10–50 mice.

The Agar Plaque Technique.—This method was used to detect individual antibody producing cells (PFC's) has previously been described in detail (18, 19).

Serological Procedures.—Hemolysin titers were determined by tube titrations except when we had only small quantities of sera available and wished to determine the content of both 7S and 19S hemolysins. Plate titrations adapted from the plaque method used to distinguish between cells producing 19S and 7S antibodies (19) were then done, using very small quantities and circumventing dialysis procedures. For each serum, four "Optilux" Petri dishes (Falcon Plastics, Los Angeles, Calif.) were supplied with a thin layer of 2 ml of 0.7% Difco agar in Eagle's solution containing 1 mg DEAE dextran and 4×10^8 sheep red cells (SRC). Volumes of 0.005 ml of undiluted serum and serial two-fold dilutions were dropped from a Carlsberg pipette onto each plate. After 30 min of incubation at 37°C, 4 ml of 0.15 M 2-mercaptoethanol (ME) were added to two plates, the remaining two receiving 4 ml of Eagle's solution. The plates were incubated 1 hr, then extensively washed to remove all trace of ME. To one control and one ME-treated plate, 1.5 ml of rabbit antiserum to mouse gamma globulin were then added. 30 min later, all four plates received 1.5 ml of complement and were incubated for a further 30 min. Reading areas showing total or partial lysis as positive, the titers obtained by this method agreed very well with those of the conventional tube method. The titer of a pure 7S preparation, developed in the presence of anti-mouse gamma globulin (indirect titer) was about 300-fold higher than that obtained in its absence (direct titer) and the titer was unaffected by treatment with ME. The same degree of potentiation by rabbit anti-mouse gamma globulin was observed in tube titrations. As shown by Humphrey and Dourmashkin (20, 21) a single antibody molecule attached to a sheep red cell can activate complement to produce lysis, whereas 600–1000 molecules of 7S antibody must be present on one sheep red cell before lysis occurs. To permit comparison on a molar basis of the effects of 7S and 19S preparations, we have defined the concentration of 19S antibody as the direct hemolytic titer of a 19S preparation and the concentration of 7S antibody as 1000 times the direct hemolytic titer of a 7S preparation.

[1] Jerne, N. K., A. A. Nordin, C. Henry, H. Fuji, and A. M. C. Koros. 1965. NIH Information Exchange Group 5. Immunopathology, Scientific Memo 46.

[2] Jerne, N. K., C. Henry, A. A. Nordin, H. Fuji, and A. M. C. Koros. The kinetics of the primary immune response and the target of the antigen. Manuscript in preparation.

Preparation of Antibody Fractions.—

7S: Serum rich in specific 7S antibody of high avidity was obtained from mice hyperimmunized by three courses of triweekly intraperitoneal injections of 4×10^8 SRC, allowing 7–10 days of rest between each course of injections. The animals were bled 10 days after the last injection. The gamma globulin was precipitated by ammonium sulfate and the excess salt removed by passage through a short column of Sephadex G-25. Good separation of 7S and 19S components was achieved when 0.3 ml was layered onto 4.5 ml of a preformed linear gradient of sucrose (ranging from 0.3–0.7 M) and centrifuged at 35,000 rpm in a SW39 rotor for 14 hr. Two fractions were harvested from each tube, and four runs gave us a pool of about 7 ml. Two such preparations were used in the experiments reported in this paper.

19S: Mice were immunized with a single intravenous injection of 4×10^8 SRC and bled 5 days later when the serum had its maximal titer of 19S antibody together with a significant amount of early 7S antibody. Three techniques were applied to isolate the macroglobulin fraction. Six 19S preparations were obtained by sucrose gradient centrifugation as described above. One preparation was obtained by Pevikon block electrophoresis followed by gel filtration on a long column of Sephadex G-200 (22). Nine preparations were made by euglobulin precipitation at pH 5.5 after the addition of 10 volumes of distilled water and storage overnight at 4°C. The precipitate was washed twice, resuspended in saline to approximately half the initial volume, and clarified by centrifugation at 3000 *g* for 30 min. The protein concentration of our nine euglobulin preparations, determined initially by a modified Folin method (23) and subsequently by absorbancy at 280 mμ ($E_{1\,cm}^{1\%}$ was calculated to be 17.5), ranged from 2–9 mg/ml. The corresponding hemolytic titers varied from 2560 to 7680. We found euglobulin to be the most convenient source of large amounts of relatively pure high titer 19S antibody. The question of contamination of the 19S preparations with specific 7S antibody will be discussed later.

Unless otherwise indicated, the mice producing the antibody and the recipient mice were of the same inbred strain, and the antibody fractions were given intravenously in a 0.1 ml volume 1–2 hr prior to the intravenous injection of SRC.

RESULTS

Experiments with 7S Antibody

*Time of Administration of 7S Antibody.—*Fig. 1 shows the PFC response of mice which had received 0.1 ml of a 7S preparation (direct lytic titer 320) before or after a single injection of 4×10^7 sheep red cells (SRC). The features displayed by this figure are in accordance with the findings reported by Möller and Wigzell (7). When the 7S antibody was given to mice in which the immune response had already been initiated by SRC given 1 day earlier, there was a delay of more than 24 hr before any inhibitory effect could be observed.

When 7S antibody is administered before the SRC, the suppression is markedly greater than when it is given 4 hr after the SRC. At that time, the SRC have already localized in the spleen.[3] Assuming that the antibody acts by combining with antigenic determinants, it would appear that either the antigen

[3] Koros, A. M. C. 1965. A study of the primary immune response, employing the technique of plaque formation in agar by single antibody-producing cells. Ph.D. dissertation. University of Pittsburgh School of Medicine. *Dissertation Abstr.* **26:** 3385.

has become protected against the effect of passively administered antibody within a few hours after SRC injection, or that the antigen has partly completed its role in the stimulus within that time period. The latter possibility would imply that antigenic stimulation is not needed for a continued rise in PFC in the spleen.

The direct titer of the passively introduced 7S antibody in these mice, assuming a 1:20 dilution in the blood, would be 16, a level never found in normal, untreated mice. Higher concentrations of 7S antibody would therefore appear to have no relevance for the early normal primary response, though ex-

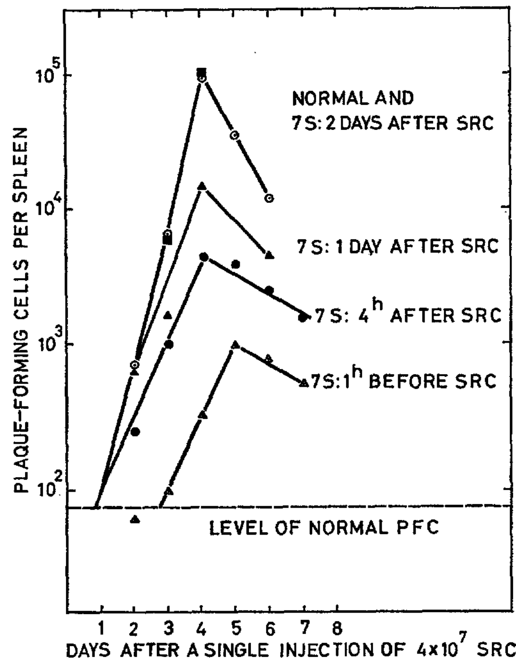

FIG. 1. Inhibition of the primary response of mice to a single intravenous injection of 4×10^7 SRC by 0.1 ml of 7S anti-SRC antibody of direct hemolytic titer 320, given intravenously. △, 1 hr before SRC; ●, 4 hr after SRC; ▲, 1 day after SRC; ■, 2 days after SRC. Controls, ⊙, received no antibody. Each point is the mean number of PFC per spleen of 10 mice.

periments with 7S preparations of high titers have yielded important information on the regulatory role of 7S antibody in later stages of the immune response (7, 10). In order to determine whether or not 7S antibody could play a role in the initiation of the normal primary response, we therefore looked for conditions in which small amounts of 7S antibody were effective.

The distribution of PFC per spleen among individuals of a group of normal mice can be described as negative binomial,[2] a distribution that can arise

under a birth and death model in which the probabilities of arrival, birth, and departure of cells are constant (24). As the birth process in untreated animals might result from a mechanism similar to that following a small dose of antigen, we tried to establish whether the maintenance of low levels of 7S antibody over an extended period might prevent the appearance of new clones of "normal" PFC in mice in the absence of antigen.

Over a 3 wk period, 30 AKR mice each received nine intravenous injections of 0.1–0.2 ml of a 7S preparation diluted to a direct hemolytic titer of 3. Assuming a half-life of 4 days for isologous 7S immunoglobulin, the times of

TABLE I

Effect of the Time of Administration of 7S Antibody on the Response of Mice to 4 $\times$ 10^8 SRC

Experimental group	Direct hemolytic titer of 7S preparation	PFC per spleen
A	—	10,000
B	(6)	320
C	12	1,400
	40	1,000
D	12	4,300
	40	2,400
E	12	4,200
	40	1,600

Group A received antigen alone. Group B received multiple injections of minute amounts of 7S antibody over a 3 wk period in an attempt to maintain, in the circulating blood, an antibody level equivalent to that resulting from a single injection of a 7S preparation of a direct hemolytic titer of 6, as described in the text. Group C received 7S antibody 1 day, and group D 1 hr prior to antigen. Group E received antibody antigen complexes. All responses were determined 3 days after the injection of SRC.

injection were spaced so that the titer of 7S antibody in the serum of the frequently injected mice would be maintained at a level similar to that produced just after a single injection of 0.1 ml of a 7S preparation of a direct lytic titer of 6. The circulatory concentration of 7S antibody introduced by such a single injection is detectable only by an indirect titration, but is sufficient to completely suppress the response to 4 $\times$ 10^6 SRC. The mean number of normal PFC per spleen of 20 of the frequently injected mice at the end of the 3 wk period was 103. In a control group assayed simultaneously, it was 47. The difference is probably negligible as the value for the control group is abnormally low. (On the average we find 80 normal PFC per spleen). There was obviously no depression of the level of normal PFC. It is possible that the

spontaneous birth and death rates of PFC in untreated mice are too low for an effect to be manifest within 3 wk.

When the remaining 10 mice of the treated group were challenged with 4×10^8 SRC and sacrificed 3 days later, they showed a mean response of only 320 PFC per spleen. An untreated control group receiving 4×10^8 SRC at the same time gave a mean response of 10,000 PFC. In Table I, this surprisingly high degree of suppression exhibited by these mice (group B) is compared with the effect of low concentrations of 7S antibody given in a single injection of 0.1 ml at various times before an injection of 4×10^8 SRC. Group E in this table received 0.1 ml of washed complexes of 4×10^8 SRC and the indicated amount of specific 7S antibody. Each value in the last column gives the response at 3 days after the injection of SRC and represents the mean of 10–20 animals. The similarity in behavior of groups D and E suggests that when 7S antibody is injected shortly before antigen, the attachment of antibody to antigen occurs in the circulation. A more effective site of action must be invoked to explain the greater suppression observed when antibody is given earlier. Since it appears that antigenic stimulation takes place in the spleen when antigen is given intravenously (25), it is possible that antibody can accumulate in crucial splenic sites. The magnitude of the suppression observed after minute amounts of 7S antibody had been administered over a 3 wk period (group B) could either be ascribed to accumulation of antibody in effective histological positions or to suppression of the emergence of antigen-sensitive target cells.

Variation of the Dose of SRC, for a Given Dose of 7S Antibody.—To investigate the inhibitory effect of a given concentration of 7S antibody on the response of mice to various doses of SRC, we selected a concentration which would have a measurable effect on a great range of antigen doses. Preliminary experiments suggested that a 7S preparation diluted to a titer of 8 by direct hemolytic test would be suitable for this purpose. A volume of 0.1 ml of this preparation was injected 1–2 hr before SRC.

Fig. 2 shows the increase with time of the mean PFC per spleen of groups of mice which had received a single injection of 4×10^7, 4×10^6, or 4×10^5 SRC. The response of the antibody-treated group given 4×10^7 SRC is very similar to that of the normal controls given 4×10^6 SRC, that of the antibody-treated group given 4×10^6 SRC similar to that of the controls given 4×10^5 SRC and the low response of the antibody-treated group given 4×10^5 SRC is similar to the normal response to 4×10^4 SRC. It thus appears that, at all SRC doses, the interaction of this concentration of 7S antibody with the sheep red cells, leaves about 10% of the determinant groups effective. This concentration of 7S antibody failed to reduce the response to 4×10^8 SRC which is compatible with this conclusion, since there is no perceptible difference in the response of normal mice injected with 4×10^8 or 4×10^7 SRC. Apparently, the

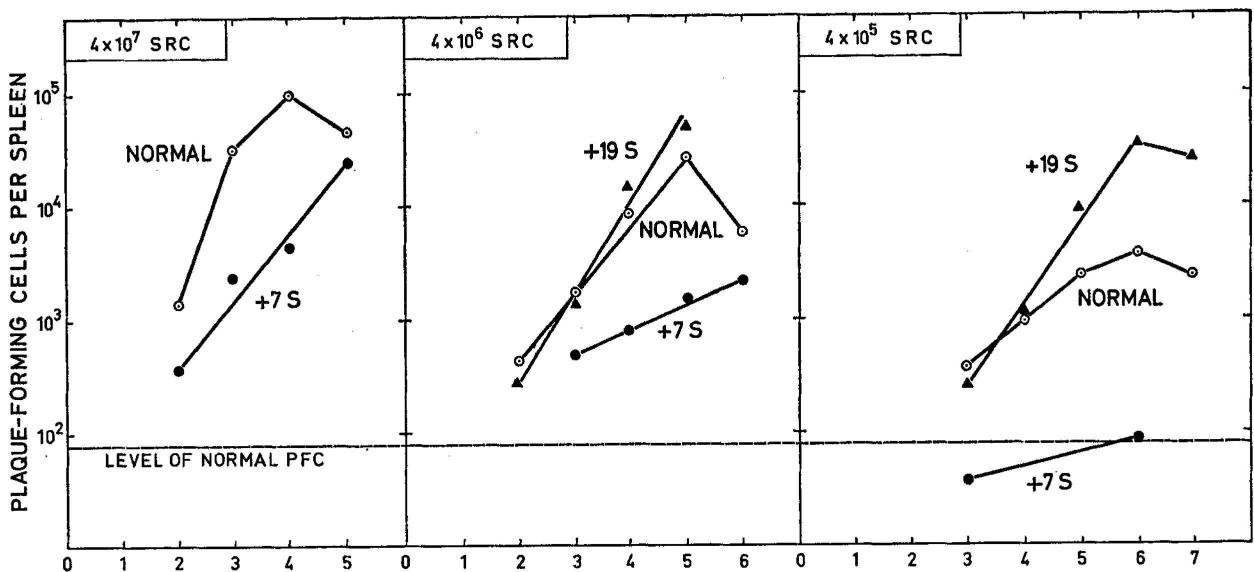

FIG. 2. Effect of a single intravenous injection of 0.1 ml of specific 7S or 19S anti-SRC antibody on the primary response of mice to 4×10^7 SRC, 4×10^6 SRC, and 4×10^5 SRC. The antibody was given 1–2 hr prior to the single intravenous injection of SRC. The 7S antibody preparation had a direct hemolytic titer of 8. The 19S antibody preparation had a hemolytic titer of 3840. ▲, response of mice that received 19S antibody. Each point represents the mean response of 20 mice. ●, response of mice that received 7S antibody. Each point represents the mean response of 10 mice. ⊙, response of control mice that received no antibody. Each point represents the mean response of 30 mice. The points plotted are the mean numbers of PFC per total spleen after deduction of the number of normal PFC shown.

antibody, even in this low concentration, is not exhausted by antigen in the dose range used. The curves denoted 19S in Fig. 2 will be discussed in a later section.

Variation in the Dose of 7S Antibody, for a Given Dose of SRC.—We have studied the effect of a single intravenous injection of different dilutions of a 7S antibody preparation (direct hemolytic titer 480) on the response of mice to 4×10^5 SRC. The mice received an injection of 0.1 ml antibody 1–2 hr before antigen, and the number of PFC's per spleen was determined 6 days later. Untreated mice injected with 4×10^5 SRC exhibit a maximum of PFC per spleen at this time. The average response at 6 days of 200 control mice that received 4×10^5 SRC was 4000 PFC per spleen. This normal response level is represented by a horizontal line in Fig. 3. The abscissa indicates, on a logarithmic scale, the concentration of passively introduced antibody in the mice, which for 7S has been calculated as 1000 $T/20$ where T is the direct hemolytic titer of the injected preparation, and a dilution of 1:20 in the blood after an intravenous injection of 0.1 ml is assumed.

The lower part of Fig. 3 shows the depressive effect of passive 7S antibody. Each solid circle is the mean number of PFC per spleen of a group of 20–50 mice 6 days after receiving 4×10^5 SRC preceded by specific 7S antibody. On the logarithmic scales shown here, the magnitude of the depression of the response appears to increase linearly with the 7S antibody concentration. Mice given higher concentrations of 7S antibody than those included in Fig. 3 did not respond to 4×10^5 SRC, whereas those given a dilution of more than 1:3000 of the 7S preparation produced a normal response level. The line drawn through the points has a slope of about one, so that the degree of 7S depression is approximately proportional to the concentration of 7S antibody.

Experiments with 19S Antibody

Variation in the Dose of 19S Antibody, for a Given Dose of SRC.—The upper part of Fig. 3 shows the response at 6 days of mice which received specific 19S antibodies 1–2 hr prior to an injection of 4×10^5 SRC. The open circles represent the responses of the groups of mice that received either undiluted or diluted euglobulin preparations. Responses of animals injected with 19S preparations obtained by density gradient centrifugation or by block electrophoresis + Sephadex G-200 are given by the triangles and the square respectively. The latter point has been encircled in Fig. 3, together with two points representing euglobulin preparations that were found, on a molar scale, to be contaminated, by about 50%, with early 7S antibody. The concentration of passively introduced 19S antibody in the mice is shown on the abscissa, calculated as $T/20$, where T is the hemolytic titer.

It will be seen that, in contrast to 7S, specific 19S anti-SRC-antibody in a wide range of concentration enhances the immune response to SRC, and that

the degree of enhancement increases linearly with the 19S concentration. A line with a slope of 0.5 has been drawn through the points, showing that the degree of 19S enhancement is approximately proportional to the square root of the 19S antibody concentration.

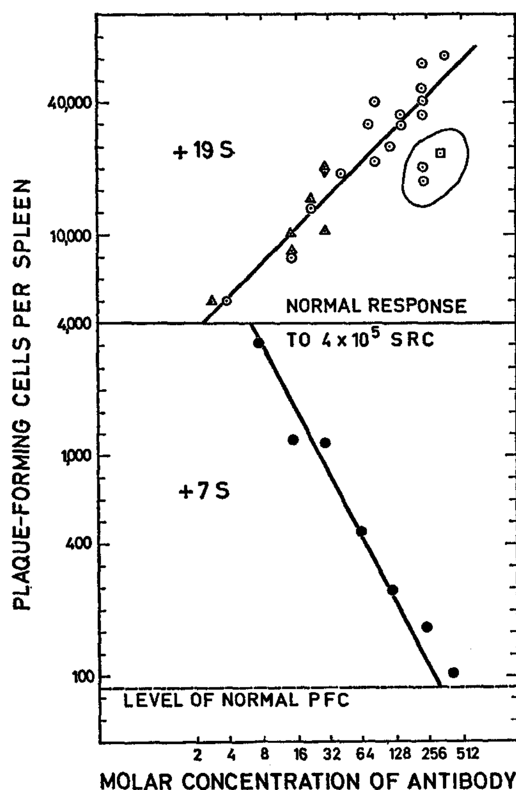

Fig. 3. Elevation by specific 19S antibody, and depression by specific 7S antibody, of the primary immune response of mice to a single intravenous injection of 4×10^5 SRC as measured by the number of PFC per total spleen 6 days later. The antibody was injected intravenously in 0.1 ml 1–2 hr before the SRC. The relative molar concentration of specific antibody was calculated from the hemolytic titer as explained in the text. The open symbols represent the average responses of groups of mice that received 19S antibody, the solid circles represent the average responses of groups that received 7S antibody. Each point is the mean number of PFC per spleen of 20–50 mice after deduction of the number of normal PFC shown.

When both curves in Fig. 3 are extrapolated to the normal response level, they denote antibody concentrations that may well be present in our normal mice. We have, in fact, found the hemolytic 19S antibody titers present in our untreated mice to range from 0–16, with a mean of 4.3. We have not, however, been able to detect antibodies of the 7S class, neither after elimination of

ME-susceptible antibodies, nor by searching for hemolytic activity in the normal 7S globulins obtained by sucrose gradient centrifugation of pooled normal mouse sera. As the potentiation of the hemolytic titer afforded by our rabbit anti-mouse gamma globulin in indirect titrations is about 300 rather than 1000, the 7S extrapolation value of Fig. 3 would be just at our limit of detection. Since evidence already presented above suggests that continued presence in the circulation may cause 7S antibody to be concentrated in critical locations, our failure to detect 7S hemolysins in the serum may not exclude a role for 7S antibody in the primary response. However, the PFC in normal mouse spleens seem to be mainly, if not exclusively, cells secreting 19S antibodies.[4]

It does not appear that the preexisting level of circulating 19S hemolysins is the only factor determining the response of an individual mouse to a SRC stimulus. If that were so, we would expect that the variation in response among mice which were passively given a high concentration of 19S hemolysin prior to 4×10^5 SRC would be less than that among untreated mice which received this dose of SRC. This has not been observed. Furthermore, we have found hardly any correlation between the level of 19S hemolysin determined in individual mice before injection of 4×10^5 SRC, and the response 6 days later. An obvious cause of the variability would be differences in the number of target cells present in individual mice. We have not discarded the idea, however, that variation in the level of preexisting specific 7S antibody may also be important.

Variations in the Time of Sacrifice and the Dose of SRC.—The enhancement by 19S antibody of the response to 4×10^5 SRC reported above was observed at 6 days after the antigen injection, the day when normal mice injected with this dose attain their peak level of PFC per spleen. Since the time/log response curves of mice not given passive antibody have decreasing slopes for decreasing antigen doses, we determined the time/log response curves of mice that had received specific 19S antibodies prior to antigen. Fig. 2 includes such curves for mice which were given 0.1 ml of a 19S preparation 1–2 hr before 4×10^5 or 4×10^6 SRC. There were 10–20 mice in each group, as well as in the corresponding control group sacrificed on the same day. The enhancing effect was not apparent during the first 3 days of the response and the 19S antibody did not appear to affect the time at which the peak response to 4×10^5 SRC was attained. Less extensive experimental results with other SRC doses are in accord with these findings.

Mice given 19S antibody and 4×10^5 SRC may, at the peak of the response, exhibit as many PFC per spleen as untreated mice given 4×10^6 SRC, but Fig. 2 shows the time curves to be markedly different. A possible interpretation is

[4] Fuji, H., and N. K. Jerne. The kinetics of the appearance of 19S and 7S antibody-releasing cells in the primary immune response. Manuscript in preparation.

that the response (as measured by PFC) of euglobulin-treated mice as well as untreated mice is similar during the first 3 days, after which the rate of increase of PFC diminishes in untreated mice but is maintained in the treated mice. It could be imagined that attachment of more 19S antibody to the red cells permits them to exert an initial stimulus sufficient to maintain a maximal rate of cell division for a longer time. Alternatively, if an initial stimulus is not sufficient to maintain cellular proliferation, the 19S-coated SRC may be so placed that it can more efficiently stimulate the progeny of the cells initially stimulated.

It can also be noted from Fig. 2 that the effect of a given dose of specific 19S antibody is less marked for the response to 4×10^6 SRC than to 4×10^5 SRC. We have investigated the degree of enhancement afforded by one 19S antibody preparation over the antigen range of 4×10^4 to 4×10^6 SRC. The mice received the 19S antibody 1–2 hr before the SRC, and they were killed when they had presumably attained their peak level of PFC, i.e., 5 days later for the doses 2×10^6 and 4×10^6 SRC, 6 days for 2×10^5, 4×10^5, and 10^6 SRC, and 8 days for 4×10^4 and 10^5 SRC. Each control and treated group contained 10 animals. Fig. 4 illustrates that the degree of elevation is considerable for the lower doses and tapers off towards high doses of SRC. This 19S preparation showed no effect on the response to 4×10^7 SRC's. A two-fold elevation of the response to this latter dose, has, however, been observed with a more potent 19S preparation, which enhanced the response to 4×10^5 SRC 15-fold and that to 4×10^6 SRC five-fold. It could be imagined that the enhancement by 19S antibody is less marked at high SRC doses because of competition with the considerable amounts of 19S (and 7S) antibody produced by the responding cells.

Identification of the Enhancing Substance.—In the previous sections, we have assumed that enhancement of the response was caused by specific 19S antibody. There are several justifications for this assumption.

Fig. 3 shows that the degree of enhancement increases with the specific hemolytic titer of the 19S preparation. In addition, it indicates that the enhancive properties of preparations obtained by three independent methods of fractionation are similar. The final product obtained by all methods contained the bulk of the hemolytic activity of mouse serum 5 days after a large dose of SRC. Sucrose gradient centrifugation selected the macroglobulins. The fraction of serum obtained after preparative zone electrophoresis followed by Sephadex G-200 filtration was the high molecular weight fraction of the components migrating in the region included by the faster part of the gamma globulin and the slower part of the beta globulin. A characterization of the euglobulin precipitate is more difficult. Human euglobulin usually contains IgM, low density lipoproteins, ceruloplasmin, beta-1C globulin, and a portion of the IgG, the composition being dependent on both pH and the ionic strength

of the medium (26). The last three components of the precipitate would, on a molecular weight basis, be selected against by the other two methods of preparation. IgM therefore seems to be the only component present in large amounts in all three types of preparation.

We have confirmed the specificity of the 19S activity. Preparations from the pooled sera of normal mice obtained either by sucrose gradient centrifugation or by euglobulin precipitation did not influence the normal response to 4×10^6

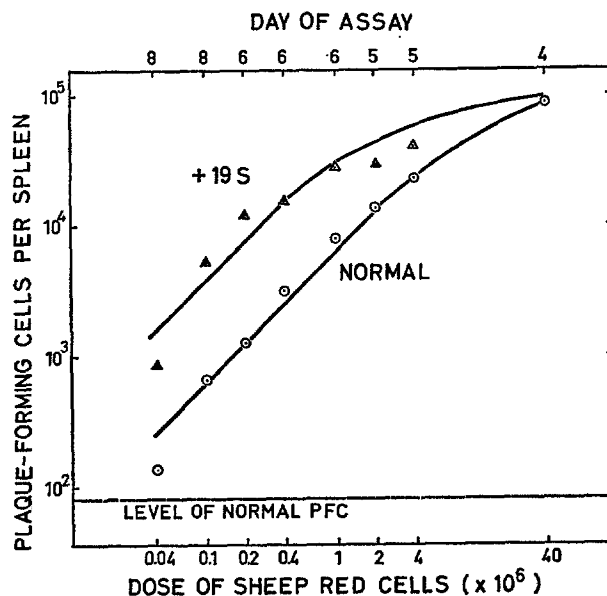

FIG. 4. Effect of a single intravenous injection of 0.1 ml of a given preparation of specific 19S antibody given 1–2 hr before a single intravenous injection of the number of SRC shown on the abscissa. The number of PFC per total spleen was determined at the day of assay indicated. The triangles represent the 19S treated mice, the circles the controls that received no antibody. Each point is the mean number of PFC per spleen of 10 mice after deduction of the number of normal PFC shown.

SRC. We have also investigated the effect of euglobulin preparations containing 19S hemolytic antibody against SRC on the response of mice to chicken and ox red cells. An anti-SRC euglobulin preparation which enhanced the response of mice to 4×10^6 SRC 10-fold was without effect on the response of mice to chicken red cells, and only marginally effective in enhancing the response of mice to ox red cells. (We observed 15% and 38% enhancement in two experiments each of which included 20 mice injected with ox red cells.) Chicken red cells and SRC cross-react to a negligible degree in mice. Spleens of mice that had been injected with ox red cells show about six times more

plaques when plated on ox red cells than when plated on SRC. In the converse situation, the number of plaques on SRC plates is 15 times that on ox red cell plates.

Are specific macroglobulins raised in animals of a different species effective in enhancing the primary response of mice? Any mechanism involving attachment of either free 19S or antigen-19S complexes to cellular receptors might require the 19S antibody to be homologous. If enhancement were the consequence of combination between specific macroglobulins and antigenic determinants, both homologous and heterologous preparations might enhance. Euglobulins obtained from NMRI mice, AKR mice, and rabbits were compared for their ability to enhance the response of NMRI mice to 4×10^5 SRC. The three preparations were obtained from sera taken 5 days after a large dose of SRC, and the amounts injected into the NMRI mice corresponded in all cases

TABLE II

Comparison of the Enhancing Effect of Specific 19S Antibody in Homologous and Heterologous Euglobulins

Source of euglobulin	PFC response per spleen of NMRI mice
None (control)	4,000
NMRI mice	31,500
AKR mice	19,000
Rabbits	10,300

Each mouse of three groups of 20 NMRI mice received 0.1 ml i.v. of a 19S antibody preparation of hemolytic titer 5120, followed 1 hr later by 4×10^5 SRC. A fourth group served as controls. PFC per spleen were determined 6 days later.

to 0.1 ml of a preparation of hemolytic titer 5120. Each test group contained 20 animals. The results are shown in Table II.

Although the passively administered heterologous 19S antibody is less effective than homologous preparations, it is unquestionably capable of enhancing the response. Species- and strain-specific properties of IgM seem to be of some consequence but not decisive for its enhancing capacity.

Purity of the 19S Preparations.—Contaminating specific 7S antibody was found in all 19S preparations by the criterium of indirect plate titration for ME-resistant hemolysins. Our method of indirect titration of 7S antibody gave values 300-fold higher than those obtained in direct titrations. Assuming that direct 7S hemolytic titers must be multiplied by 1000 for molar comparison with 19S hemolytic titers, we have multiplied the indirect ME-resistant titers by 10/3. On this presumed molar basis, we have estimated that about 5% of the specific antibody molecules in the density gradient preparations of 19S were 7S antibody whereas most euglobulin preparations and the preparation

obtained by electrophoresis + Sephadex G-200 showed 15–25% contamination with specific 7S globulin. This degree of contamination has been ignored (as was done in Table III) because of the low avidity of the contaminating early 7S antibody. Several workers have shown that 7S antibody of low avidity is less suppressive than more avid antibody. In agreement with these findings,

TABLE III

Responses of Mice Injected with 19S and 7S Antibodies, and Mixtures Thereof, Prior to 4 × 10⁵ SRC

Hemolytic titer of 19S antibody preparation (*A*)	Hemolytic titer of 7S antibody preparation (*B*)	Total PFC per spleen	
		Observed	Calculated*
—	—	3,600	4,000
7680	—	54,000	35,000
3840	—	37,000	29,500
1920	—	16,800	22,500
960	—	10,500	16,000
—	9.6	92	90
—	4.8	160	170
—	2.4	242	330
—	1.2	450	620
—	0.6	1,125	1,100
—	0.3	1,150	1,700
7680	9.6	1,500	3,250
3840	4.8	2,400	3,300
3840	2.4	6,600	6,000
3840	1.2	13,400	10,000
3840	0.6	14,000	14,500
1920	1.2	8,000	5,800
1920	0.6	9,400	9,200
960	1.2	1,400	3,600
960	0.6	6,400	5,600

* See Appendix.

The figures in the first two columns represent the direct hemolytic titers of the antibody preparations injected. The amount of each preparation injected was 0.1 ml in all cases. The first seven groups contained 20 mice each, the remainder contained 10 each. The responses were determined 6 days after the injection of SRC's.

we have found that the enhancing capability of our euglobulin preparations can be reduced by the addition of an amount of hyperimmune 7S antibody which is many times lower than that of the low avidity early 7S antibody already present in the 19S preparation. The contaminating low avidity 7S antibody is perceptibly depressive, however, when the level of contamination exceeds about 20%. Two euglobulin preparations, which showed about 50% contamination with specific early 7S antibody, were only half as effective in

enhancing the response to 4×10^5 SRC as would be predicted on the basis of their 19S hemolytic titers. The effect of these two preparations is represented by the two encircled points which lie significantly beneath the 19S curve in Fig. 3. Repeated precipitation in aqueous solution at pH 5.5 yielded 19S antibody preparations of greater purity but resulted in considerable loss of macroglobulin. The contamination of our 19S preparations with specific "7S" antibody was calculated above on the basis of determinations of the presence of ME-resistant hemolytic antibody. The degree of contamination seems quite high and probably does not represent a contamination with 7S molecules but with polymerized IgG molecules.

Experiments with Mixtures of 19S and 7S Antibodies

When mixtures of 19S and 7S antibodies are injected prior to SRC, the response is intermediate between the enhanced value and the depressed value observed when either type of preparation is given alone. Groups of 10–20 NMRI mice received 4×10^5 SRC intravenously, preceded 1–2 hr earlier by an intravenous injection of 0.1 ml of a 19S antibody preparation, or a 7S antibody preparation, or a mixture of the two. In the first two columns of Table III, the 19S hemolytic titer (A) and the direct 7S hemolytic titer (B) of the antibody injected is given. The third column of this table shows the number of PFC per spleen determined 6 days later. For example, it is seen that an almost normal response level is obtained when a 19S antibody preparation of hemolytic titer 3840 is given together with a 7S antibody preparation of direct hemolytic titer 4.8. We have tentatively explored a model for this 7S versus 19S antagonism. The model, which is described in the Appendix to this paper, is based on a competition of 7S and 19S antibody molecules for available sites on the SRC surface and proposes that only antigenic determinants that are covered by a 19S antibody molecule can stimulate a target lymphocyte.

DISCUSSION

The present experiments contribute to a subject that is coming into the focus of attention in immunology: the question of the multiplicity of antigen receptors. It has been clear for a long time (27) that, before the events leading to antibody formation can be initiated, the antigen must be recognized as an immunogenic particle. To this end, antigen receptors must be available and these must exhibit antibody-combining sites. Plainly speaking, they must be antibody molecules. One type of antigen receptors may be situated on the target lymphocytes. The experiments on blast transformation of small lymphocytes by Sell and Gell (28), as well as those on allotypic suppression by Dubiski and Fradette (29), Mage and Dray (30) offer evidence for the existance of such lymphocytic receptors which have properties in common with immunoglobulin molecules. It would be most simple to suppose that a cell is triggered to produce

antibody and to proliferate by a direct encounter of circulating antigen with the antigen receptors of this cell. The mechanism appears to be more complex, however. The experiments of Mitchison (31), Benacerraf (32), Rajewsky and Rottländer (33), and others appear to have established the fact that immunogenicity of an antigenic particle involves at least two properties: a "carrier" property and a "hapten" property. It would seem that unless carrier sites on the antigen are recognized by an early antigen receptor, the haptenic sites cannot trigger the final antigen receptor of the target lymphocyte. In order to resolve these questions we need to clarify the number of different types of antigen receptors, as well as their function. An obvious class of antigen receptors which participate in the events leading to an immune response, or which can intervene in these events, are the circulating antibody molecules themselves, both those that may have arisen spontaneously as "natural" antibodies and those that owe their existence to a previous antigenic stimulus. It is therefore necessary to investigate the effect of antibodies of all immunoglobulin classes in the primary and secondary immune response.

The results presented in the present paper show that the feedback effect of antibodies on the primary immune response is not always negative. We have confirmed that the specific antibody contained in 7S fractions suppresses the primary immune response. We have demonstrated that the antibody contained in 19S fractions enhances the primary response of mice to SRC. Our most concentrated preparations of 19S antibody, when given prior to a moderate dose of SRC, increased the response 10- to 15-fold.

Contrary findings by others that 19S antibody is suppressive may perhaps be explained by a contamination of 19S preparations by 7S antibody. With current methods of fractionation it is difficult to obtain sizable quantities of pure 19S immunoglobulin. As 7S antibody is an inefficient hemolytic antibody compared to 19S antibody, a considerable contamination on a molar scale of comparison may go unnoticed. Furthermore, as we have shown that the degree of depression increases more rapidly with increasing concentrations of 7S antibody than the degree of enhancement rises with increasing concentrations of 19S antibody, contaminating 7S antibody will be less effective in small doses of contaminated preparations, but may dominate in larger doses.

In our experiments with specific 7S antibody, we have focussed attention on the fact that even very low concentrations of 7S antibody suffice for a marked suppression of the primary response. Depression is more pronounced when more time is allowed between the injection of 7S antibody and the injection of the antigen. It is reasonable to believe that the depressive effect operates also in the normal response when 7S antibodies of sufficient avidity make their appearance. The enhancing effect of 19S antibodies likewise must be assumed to play a role in the normal response, since 19S antibodies are the first to be produced and, moreover, can be demonstrated prior to the antigenic stimulus as "natural" antibodies.

In reviewing our experimental results, it seems striking that in many cases there is a time span between the injection of antibody and the first signs of an effect. Thus 7S antibody in several of our experiments, as well as in those of Möller and Wigzell (7) and Wigzell (10) fails to manifest its effect until 24–48 hr after injection. Likewise, the presence of 19S antibody does not markedly affect the number of PFC arising in the early phase after an antigenic stimulus, but manifests its effect after the 3rd day.

It is important to note that antibodies harvested from one animal species are active in a different animal species, as this suggests that the mechanism by which the antibody acts is not at a genetic or synthetic level but rather involves the reaction of antibody-combining sites with the antigen and perhaps also the cytophilic properties of the antibody molecules. It is most simple to imagine that the suppressive effect of 7S antibody molecules results from coverage of antigenic determinants which are thus prevented from participating in the immunogenic stimulus. It is more difficult to suggest a mechanism for enhancement by 19S antibody. A possibility that comes to mind is that 19S molecules, attached cytophilically to macrophage membranes, may capture antigenic particles by combining with antigenic determinants and thereby offer other antigenic determinants that have remained free to the target lymphocytes.

It remains to be seen whether antibody molecules of certain Ig classes are necessary components of the primary immune stimulus, and to what extent other Ig classes of antibody can interfere.

SUMMARY

Prior to sheep red cells (SRC) mice were given 7S or 19S anti-SRC antibodies or mixtures of both. All 7S preparations suppressed the immune response. All 19S preparations enhanced the primary response, as measured by an up to 15-fold increase in the number of PFC per spleen. Results obtained with mixtures showed that 7S and 19S antibodies are competitive in their effect. The kinetics of the appearance of PFC in the mouse spleen after injection of SRC suggest that the depressive effect of 7S antibody simulates a reduction in SRC dose, whereas the enhancing effect of 19S antibody appears as a temporary increase in the rate at which PFC appear. Antibodies from one animal species are quite effective in another species.

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APPENDIX

A model, suggested by Dr. D. Kodlin, assumes that the maximal yield of antibody-forming cells (measured as PFC) after the injection of a given number of SRC is proportional to the average number of 19S antibody molecules attached to a SRC. Specific 7S and 19S antibody molecules compete for sites on the SRC, and the depressive action of 7S antibody is explained by the reduction of the fraction of sites that remain occupied by 19S molecules.

Assuming the reversible equilibrium

$$\text{site} + \text{antibody molecule} \rightleftharpoons \text{occupied site}$$

it can be shown that the fraction F of sites to which 19S antibody molecules are attached is

$$F = \frac{K_a a}{1 + K_a a + K_b b}$$

in which K_a and K_b are the association constants of the 19S and 7S antibody with SRC determinants, and a and b the molar concentrations of 19S and 7S antibody. Considering that in normal mice a small concentration of 19S hemolytic antibody, but not of 7S antibody, can be detected, the following parameters were adopted:

$$K_a = 10^9 \text{ M}^{-1}$$

$$K_b = 10^{10} \text{ M}^{-1}$$

$$a = 10^{-10} + 0.05 \times 10^{-11} \times A \text{ M}$$

$$b = 0.05 \times 10^{-8} \times B \text{ M}$$

where A and B stand for the direct hemolytic titers of the 19S and 7S antibody administered in a 0.1 ml volume. The association constants adopted lie within the range found for human blood group antibodies (34). The factor 0.05 reflects the dilution of 0.1 ml into the blood of a mouse. The lowest concentrations detectable by direct hemolytic tests are assumed to be 10^{-8} M for IgG and 10^{-11} M for IgM.

Using these parameters, the formula shows that in normal mice $F_0 = 0.09$, i.e. that on the average 9% of the sites of injected SRC become occupied by 19S antibody molecules. As normal mice show an average response to 4×10^5 SRC of 4000 PFC per spleen 6 days after the SRC injection, it is postulated that antibody-treated mice will show an average response of $F/F_0 \times 4000$ PFC.

In Table III the hemolytic titers A and B of the antibody mixture injected have been listed, as well as the response values calculated by this model. It is seen that these agree satisfactorily with the experimental results. This agreement may be merely accidental, however, and the model does not account for the apparent differences in mode of action of 19S and 7S antibodies, that we have described.

A relationship of the same type as given by the above formula would result from a model which assumes that the response is directly proportional both to the concentration a of specific IgM and to the fraction $1/f$ of antigenic determinants remaining free of antibody attachment ($f = 1 + K_a a + K_b b$).