REGULATION OF ANTIBODY FORMATION BY SERUM ANTIBODY

II. REMOVAL OF SPECIFIC ANTIBODY BY MEANS OF EXCHANGE TRANSFUSION*

BY JEAN-CLAUDE BYSTRYN,‡ M.D., MARTIN W. GRAF, M.D., AND JONATHAN W. UHR, M.D.

(From the Irvington House Institute and the Department of Medicine, New York University School of Medicine, New York 10016)

In an earlier study from this laboratory (1), it was shown that serum antibody could be removed specifically from immunized animals by exposing their plasma to an immunoadsorbent and returning the adsorbed plasma to them. 1 or 2 days after the removal, serum antibody levels began to rise, reaching a peak 5 days later. The question of whether antigen released from the immunoadsorbent and returned to the circulation of the animal was responsible for the response was considered. Evidence was obtained that only trace amounts of antigen were released from the immunoadsorbent and that these amounts were insufficient to account for the response.

Because of the critical nature of this question, however, we have attempted to remove antibody from immunized animals under circumstances in which re-exposure to exogenous antigen could not occur. In addition, it was planned to study, by passive antibody administration, the extent to which redistribution of extravascular antibody affects the rise in levels of serum antibody after its removal. The experimental approach used was to immunize rabbits to two antigens and later to exchange their blood with that of rabbits immunized to only one of the antigens. In this way, antibody levels to one antigen were maintained, whereas antibody levels to the second antigen were markedly reduced. The results confirm and extend those previously reported: Following removal of antibody, specific antibody levels began to rise and in every case reached levels above those present before the exchange. In contrast, only a small increase in antibody levels was observed when antibody was passively administered before exchange.

Materials and Methods

Antigens.—Bacteriophage ϕX 174 (ϕX) was a gift from Dr. R. Sinsheimer, bacteriophage T_2 (T_2) was obtained from Miles Laboratories Inc. (Elkhart, Ind.), and bacteriophage T_3 (T_3) was prepared by the method of Adams (2). Bovine serum albumin (BSA), 4X recrystallized was purchased from Nutritional Biochemical Corp. Cleveland, Ohio.

Immunization.—Albino rabbits weighing 3.5–4.5 kg were used. For active immunization, antigens were given intravenously into the ear in 1–2 ml of phosphate buffered saline: 5×10^{10} ϕX, 3–7 $\times 10^{10}$ T_2, 1.5 $\times 10^{10}$ plaque-forming units T_3, or 1 mg/kg BSA.

For passive immunization, 2–6 ml of rabbit anti-ϕX (k of 500) or 12 ml rabbit anti-BSA (1500 μg anti-BSA/ml) were injected intravenously.

Antibody Assays.—Antibodies to bacteriophage were determined by the neutralization method described by Adams (2). Anti-BSA was determined by the Farr technique using 0.4 μg antigen nitrogen and an antigen-binding capacity precipitating line of 33% (3).

Exchange Transfusion.—Groups of four rabbits were immunized with one antigen (Fig. 1). One rabbit in each group, the prospective recipient, was immunized with a second antigen. 1 day before the exchange, antibody titers were determined in the recipient and prospective donor rabbits.[1] The next morning, the two donors with antibody titers nearest to that of the recipient's were injected with 1000 units/kg of heparin and bled by intracardiac puncture. A total of 200–250 ml of blood was collected into a 250 ml Blood-Pack (Fenwal Laboratories, Morton Grove, Ill.) containing acid citrate dextrose as an anticoagulant. The blood was kept at 4°C until ready for use. The recipient animal was injected intravenously with heparin 1000 units/kg and atropine 2 mg, followed 30 min later by Nembutal 30 mg/kg. The femoral vein and artery were cannulated with 16 gauge medium Intracaths (R. C. Bard, Murray Hill, N. J.), each fitted with a three-way stopcock. Blood was removed with a syringe in 40 ml portions and immediately replaced by donors' blood administered through a Hemoset (Fenwal Laboratories). A total of 200–250 ml of blood was so exchanged in less then 40 min. Immediately after exchange and 24 hr later, the animal was injected intramuscularly with 4 mg Coumadin and 1 ml Wycillin S-M.

Separation of IgG from IgM by Sucrose Gradient Ultracentrifugation.—0.2 ml samples of sera were diluted with 0.2 ml of 10% sucrose, layered on a 10–40% discontinuous sucrose gradient (4), and centrifuged at 35,000 rpm in a SW-39 rotor in a Beckman Model L ultracentrifuge for 18 hr. Immunodiffusion studies had shown that IgM was present in the bottom 3 of 15 fractions collected, whereas IgG was present in fractions 6 through 12. Fractions 1–4 (containing IgM) and 5–15 (containing IgG) were separately pooled. The pooled fractions were dialyzed for 12 hr at 4°C against 200 volumes of phosphate buffered saline pH 7.3 before antibody assays were performed.

RESULTS

Effect of Exchange Transfusion on Serum Antibody Levels.—As shown in Table I, eight rabbits were immunized to two antigens and 3–6 wk later, when antibody titers had reached a plateau or were declining, an attempt was made to remove antibody to only one of the antigens by exchange transfusion. To determine the contribution of antibody redistribution from the extravascular space to changes in posttransfusion antibody levels, four of the above animals were injected intravenously with antibody to a third, unrelated antigen 4–7

---

[1] Typing and crossmatching of blood from potential donors and recipients was performed for the earlier experiments but was discontinued when no incompatibilities were observed between the first 12 rabbits.

days before removal of antibody. Antibody titers were determined at frequent intervals before and after exchange transfusion.

The serum antibody levels of four representative animals are illustrated in Fig. 2. As can be seen in Fig. 2 a, 80% of antibody to T_2 was removed by ex-
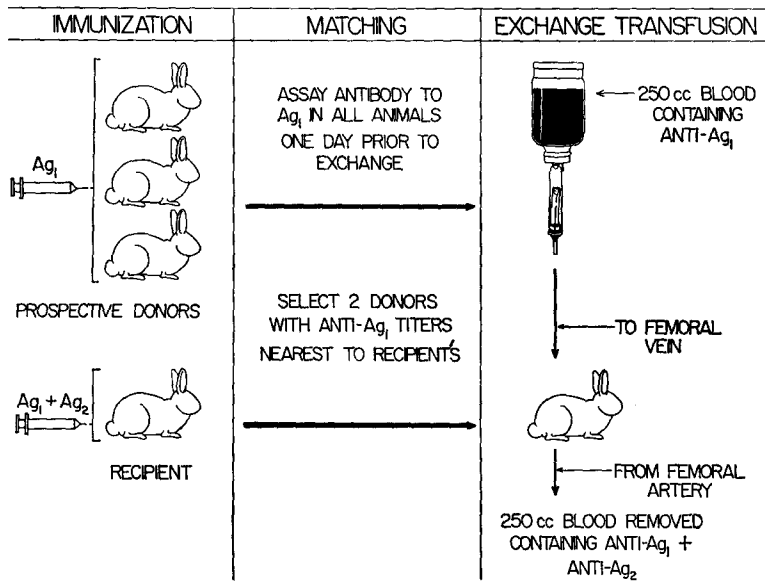

FIG. 1. Removal of antibody by exchange transfusion

TABLE I

Immunization Schedule for Rabbits

| Animal No. | Active immunization | | Passive immunization |
	Antibody removed	Antibody not removed (control)	
CM 6	anti-ϕX	anti-T_2	—
CM 7	"	"	—
CM 8	"	"	—
B 17	anti-T_2	anti-ϕX	—
B 21	"	"	anti-BSA
B 25	"	anti-BSA	anti-ϕX
B 36	"	anti-T_3	"
B 40	anti-T_3	anti-T_2	"

— = not done.

change transfusion whereas that to ϕX was increased by approximately 50%. During the next 48 hr, there was a rapid increase in antibody levels to T_2 followed by a slower rise of serum antibody reaching a peak 7 days later. Peak titers were approximately 50% above preexchange levels. In contrast, antibody

levels to ϕX declined slowly. In this animal, antibody to BSA had been passively administered 4 days before exchange. Approximately 80% of antibody to BSA was removed. A small increase in titer occurred within the next 24 hr. Fig. 2 b shows that removal of anti-T_3 was followed by a biphasic rise in anti-T_3 titers analogous to that just described in Fig. 2 a. Inadvertently, the control
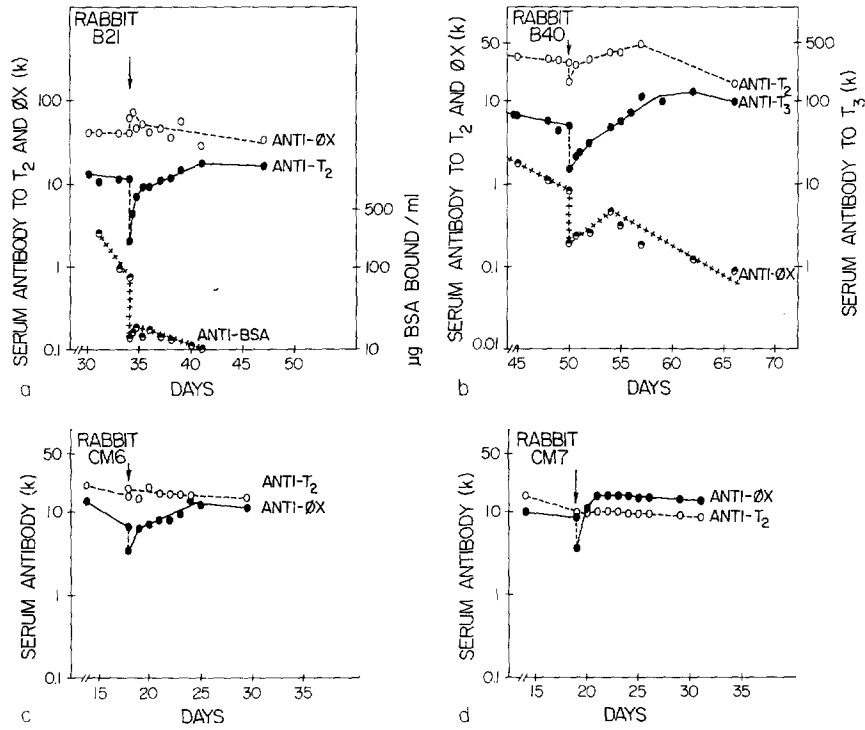

Fig. 2. Serum antibody levels in immunized rabbits before and after exchange transfusion. Four individual rabbits each immunized to two antigens are shown. Exchange transfusion with blood from rabbits immunized to one antigen only was performed on the day designated by the arrow. Rabbits B21 and B40, also passively received antibody to a third antigen 4–7 days before exchange. ●——● antibody removed by exchange transfusion; O----O control antibody; ●xxxxxxx● passive antibody.

antibody to T_2 was lowered 40% (because the donor's serum antibody level was not properly matched with that of the recipient) and a small increase was also seen in anti-T_2 levels. Anti-ϕX, passively administered, showed a significant equilibration reaching approximately 50% of the preexchange level 96 hr after removal. Fig. 2 c shows that 50% of serum antibody activity to ϕX was removed whereas anti-T_2 titers were slightly elevated. Antibody levels to ϕX rose rapidly so that the titer 1 day later was almost equivalent to the preexchange titer;

later it rose more slowly so that peak levels were reached 1 wk later, at which time the serum antibody level was approximately twice that of the preexchange titer. Antibody levels to T_2 continued stationary. Fig. 2 d shows a response that was seen in two of three animals in which antibody to ϕX was removed: a rapid rise of serum antibody levels reaching the preexchange titer in 24 hr and the peak level in 48 hr. These three animals had been immunized 18 days previously, in contrast to the remaining five animals which had been immunized 35–55 days previously.

Table II summarizes the percentage change in antibody levels resulting from

TABLE II

Changes in Serum Antibody Levels Resulting from Exchange Transfusion

Animal No.	Test antibody			Passive antibody			Control antibody	
	Serum antibody level (% of pre-exchange titer)		Interval between transfusion and peak of rebound	Serum antibody level (% of preex-change titer)		Interval between transfusion and peak of rebound	Serum antibody level (% of preex-change titer)	
	Post-exchange	Peak post-exchange		Post-exchange	Peak post-exchange		Post-exchange	8 days post-exchange
			days			*days*		
CM 6	−50	+100	6	—	—	—	+20	0
CM 7	−58	+80	2	—	—	—	−8	−6
CM 8	−68	+54	3	—	—	—	+5	−5
B 17	−84	+82	7	—	—	—	+23	−60
B 21	−83	+48	7	−82	−76	1	+48	−33
B 25	−75	+222	19	−79	−67	1.5	−32	−20
B 36	−50	+75	7	−71	−54	1	−19	−11
B 40	−70	+130	12	−77	−59	4	−41	+42
Average	−67%	+100%	8	−77%	−64%	2	−0.5%	−22%

— = not done.

exchange transfusion in all eight rabbits. 50–84% of serum antibody in actively immunized animals could be removed by the exchange. In each animal, there followed a marked increase in titer (rebound). At the peak of the rebound titers were 48–222% above the preexchange ones, even though the exchange had been performed at a time when antibody levels were at a plateau or declining. Peak titers were reached in 6–19 days after exchange transfusion in six of the animals, and in 2-3 days in the other two animals. As expected, passive antibody was removed as efficiently as synthesized antibody (71–82%). In contrast however only a small rebound resulted. Thus, peak titers which occurred usually within 48 hr were 54–76% below their preexchange levels. Control antibody levels in seven of eight animals showed no rebound.

These experiments show that (a) serum levels of antibody can be rapidly,

specifically, and profoundly decreased by exchange transfusion; (b) following exchange in actively immunized animals, there is an increase in serum levels of the corresponding antibody only; (c) this rebound achieves higher titers, and peak titer occurs later than the rebound caused by redistribution of passive antibody.

Distribution of Antibody Classes Before and After Exchange.—Because low concentrations of IgM antibody to bacteriophage can inhibit the rate of neutralization by IgG antibodies (5), we determined whether alterations in the ratio of IgM to IgG antibodies following exchange transfusion were responsible for the increased antibody titers. Antibody titrations were performed after ultracentrifugation in a sucrose gradient or after treatment with 2-mercaptoethanol (2-ME). Treatment with 2-ME destroys the bacteriophage neutralizing activity of rabbit IgM but not IgG antibody (6).

TABLE III

Changes in Serum 7S and 19S Antibody Levels Resulting from Exchange Transfusion

Animal No.	Serum antibody (k)							
	Untreated		After treatment with mercaptoethanol		Sucrose gradient fractions			
					7S		19S	
	Pre-exchange	Peak post-exchange	Pre-exchange	Peak post-exchange	Pre-exchange	Peak post-exchange	Pre-exchange	Peak post-exchange
B 17	3.0	5.5	12	17.5	3.5	6.2	0.33	0.09
B 21	11.8	17.5	23	65	16.4	29	0.75	1.56
B 25	12.7	42	26	37	17.2	25	0.67	0.19
B 36	26	46	33	47	12	18.2	0.41	0.21
B 40	50	128	55	86	32	49	0.18	0.12

The results are summarized in Table III. In 8 of 10 sera tested, treatment with 2-ME increased the neutralizing activity (k) suggesting that IgM antibody was inhibiting IgG antibody. However, in each animal there was a higher titer of both 7S- and 2-ME-resistant antibody in the postexchange compared to the preexchange sample. In contrast, 19S antibody fell after exchange in four of five animals and in all five animals represented a minor component of the neutralizing activity.

These results indicate, therefore, that the increased titer of the rebound is due to 7S (presumably IgG) antibody with little or no participation of IgM.

DISCUSSION

These studies confirm and extend results of our previous "subtraction" experiments in which serum antibody was removed specifically from immunized rabbits by exposing their blood or plasma to an immunoadsorbent (1). The

present studies indicate that the concentration of a serum antibody can be specifically and efficiently decreased by exchange transfusion. Antibody levels can also be lowered by plasmapheresis (7, 8), but for our experiments it was essential that the level of antibody of a single specificity be decreased so that the immunological specificity of the regulatory mechanism could be established. Using rabbits immunized to two antigens and exchanging their blood with blood containing one antibody only, as much as 85% of intravascular antibody of one specificity could be removed in 30 min without greatly affecting the titers of the second antibody. We could, therefore, study the results of specific antibody deletion without concern about reexposure to exogenous antigen released from an immunoadsorbent. Following exchange transfusion there was a biphasic rise in serum antibody. There was an initial rapid increase reaching levels 50–80% of the preexchange ones during the first 48 hr, followed by a slow and sustained rise which reached peak titer 1–2½ wk later. In each case the peak titer exceeded the preexchange titer by 54–222%.

We have considered several possible explanations for this rebound: (a) nonspecific change in Ig concentration, (b) redistribution of antibody, (c) change in the quality of antibody, (d) increase in rate of antibody synthesis.

Nonspecific increase in Ig concentration could result from a decreased rate of Ig catabolism, an increased rate of Ig synthesis, or from hemoconcentration. This possibility can be excluded because when levels of control antibody were maintained, no increase of these levels occurred.

The contribution of redistribution of extravascular antibody to the rebound was investigated. Previous studies (9, 10) have shown that after equilibration, approximately 50% of IgG and 20% of IgM injected intravenously into rabbits is in the extravascular compartment(s). Hence, after rapid removal of intravascular antibody, serum antibody levels would be expected to rise due to reequilibration. By removal of passively administered antibody, the kinetics of subsequent serum antibody increase caused by redistribution of extravascular antibody was determined. In confirmation of earlier studies by Gitlin and Janeway (11) the peak of rebound usually occurred within 48 hr and was small compared to that observed in actively immunized animals. Hence, redistribution of preformed antibody by itself is a minor contribution to the rebound.

Removal of serum antibody may change the quality of antibody in the circulation. Since the vast majority of total body IgM is intravascular in contrast to IgG, removal of intravascular antibody preferentially depletes IgM antibody from the host. Hence, after reequilibration has occurred between intra- and extravascular compartments, the proportion of IgG to IgM antibodies in the circulation may be altered. This possibility was important to investigate because of the known capacity of IgM antibody to bacteriophage to inhibit the rate of neutralization of phage by IgG antibody (5). We have excluded this possibility by demonstrating that the higher antibody levels of the postexchange

compared to the preexchange sera were still present after IgM antibodies had been either removed by ultracentrifugation in a sucrose gradient or inactivated by treatment with 2-ME. These studies also showed that virtually all neutralizing activity in the above sera was due to 7S (presumably IgG) antibodies. Competition between 7S antibodies has not been described. Another possibility to be considered is whether a change in average binding affinity of antibody has occurred and contributed significantly to the increase in neutralizing activity. Thus it could be argued that phage neutralization measures preferentially high affinity antibody (12), and that removal of antibody and continued synthesis increases the proportion of newly formed and hence high affinity antibody. Differences in avidity (dissociation of antibody from specific complexes) to viruses with time after immunization have been reported (13–15) but are not easily detected at this stage of immunization over a period of 2 wk.

An increase in the rate of antibody formation following exchange is consistent with the kinetics of the rise in serum antibody levels and the high peak titer achieved. This interpretation implies that serum antibody has inhibited continued immunization by endogenous antigen which is known to persist for prolonged periods of time (16), and that after removal of serum antibody persisting antigen has become more immunogenic. We favor this explanation but also consider the possibility that a change in the proportion of antibodies of different binding affinities may be contributing to the extremely high titers of antibody after the exchange and hence account for the apparent "overshoot" in the homeostatic mechanism.

SUMMARY

Rabbits were immunized to two antigens and 18-55 days later exchange transfusion was performed using blood of rabbits immunized to one antigen only. By this means, serum antibody levels to one antigen were reduced 50-84% while maintaining serum antibody levels to the second antigen. After exchange, serum antibody levels of the removed antibody rose rapidly for 24–48 hr and then more slowly, reaching peak titers an average of 8 days later. The peak titer was 48–222% higher than the preexchange titer. The specificity of this rebound excluded as a cause nonspecific changes in Ig levels. Passive administration of antibody to a third antigen 4–7 days before the exchange indicated that reequilibration of preformed antibody was not a major factor in the rebound. A change in the ratio of IgM to IgG antibodies as a cause of an increased neutralization titer in the postexchange sera was also excluded. It was therefore suggested that a change in the rate of antibody formation had occurred, although other changes in the quality of serum antibody were not excluded.

BIBLIOGRAPHY

1. Graf, M. W., and J. W. Uhr. 1969. Regulation of antibody formation by serum antibody. I. Removal of specific antibody by means of immunoadsorption. *J. Exp. Med.* **130:**1175.
2. Adams, M. H. 1959. *In* Bacteriophages. Interscience Publishers, Inc., New York.
3. Farr, R. S. 1958. A quantitative immunochemical measure of the primary interaction between I-BSA and antibody. *J. Infect. Dis.* **103:**239.
4. Williams, C. A., and M. W. Chase. 1968. *In* Methods in Immunology and Immunochemistry. Academic Press, Inc., New York. **2:**103.
5. Turner, K. J., and V. Krishnapillai. 1968. The influence of IgM antibody on the neutralization of T₄r bacteriophage by IgG antibody. *Immunochemistry.* **5:**9.
6. Bauer, D. C., and A. B. Stavitsky. 1961. 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. Sci. U.S.A.* **47:**1667.
7. Smolens, J., J. J. Stokes, and A. B. Vogt. 1957. Human plasmapheresis and its effect on antibodies. *J. Immunol.* **79:**434.
8. Smolens, J., and J. J. Stokes. 1966. The effect of plasmapheresis on plasma proteins. Proceedings 20th Scientific Meeting of the Protein Foundation, Boston, Mass.
9. Taliaferro, W. H., and D. W. Talmage. 1956. Antibodies in the rabbit with different rates of metabolic decay. *J. Infect. Dis.* **99:**21.
10. Waldmann, T. A., and W. Strober. 1969. Metabolism of immunoglobulins. *Progr. Allergy.* **13:**1.
11. Gitlin, D., and C. A. Janeway. 1953. The dynamic equilibrium between circulating and extravascular plasma proteins. *Science (Washington).* **118:**301.
12. Krummel, W. H., and J. W. Uhr. 1969. A mathematical and experimental study of the kinetics of neutralization of bacteriophage φX 174 by antibodies. *J. Immunol.* **102:**772.
13. Jerne, N. K., and P. J. Avegno. 1956. The development of the phage inactivating properties of serum during the course of specific immunization of an animal: reversible and irreversible inactivation. *J. Immunol.* **76:**200.
14. Svehag, S. E. 1965. The formation and properties of poliovirus neutralizing antibody. V. Changes in the qualities of 19S and 7S rabbit antibodies following immunization. *Acta Pathol. Microbiol. Scand.* **64:**103.
15. Finkelstein, M. S., and J. W. Uhr. 1966. Antibody formation. V. The avidity of γM and γG guinea pig antibodies to bacteriophage φX 174. *J. Immunol.* **97:**565.
16. Campbell, O. H., and J. S. Garvey. 1963. Nature of retained antigen and its role in immune mechanisms. *Advan. Immunol.* **3:**261.