rabbit skin for the Schwartzman reaction in doses of 250 μg. Pyrogenicity of the material was not tested. Serologic tests using the Ouchterlony technic are presented in Fig. 1.

Whereas there were several antigenic components in the crude material, only a single antigenic component was present in the purified antigen tested. Further, it was evident that the Vi antigen from *S. paratyphi C* was identical with that isolated from *S. typhosa*. Similar tests not presented here revealed an identity of the paratyphi C Vi antigen with those isolated from *E. coli* and *P. ballerup*.

*Summary and conclusions.* A method has been outlined for isolation and purification of the Vi antigen produced by *S. paratyphi C*. Consideration of both the chemical biological properties of this antigen indicated that it is very similar to, if not identical with, those produced by other *Enterobacteriaceae*.

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## Elimination of Circulating Serum Protein Antigens as a Sensitive Measure of Antibody.* (26101)

ROY PATTERSON,† WILLIAM O. WEIGLE,‡ AND FRANK J. DIXON
*Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pa.*

The production of antibody following injection of soluble serum protein antigens into animals can be readily detected by the rapid elimination of the antigen from the circulation of the sensitized animal(1,2). This rapid elimination presumably results from the reaction of the circulating antigen with the newly synthesized antibody and uptake of the complex by phagocytic cells(1). Rate of elimination depends on amount of antigen injected, rate of antibody formation and perhaps quality of antibody produced(3). The accelerated elimination of serum protein antigens from the circulation also takes place following both injection of antigen in actively (4,5) and passively(4,6,7) immunized animals and injection of insoluble(8,9) and certain soluble(8) antigen-antibody complexes into normal animals. The purpose of the present investigation was to determine the minimum amount of passively transferred antibody which could be detected by the rapid elimination of $I^{131}$ labeled antigen. The possibility of utilizing the rapid elimination of soluble antigens as a method to measure small amounts of antibody is discussed.

*Methods and materials. $I^{131}$ labeled antigens.* Bovine serum albumin (BSA), Armour and Co., Lot No. S 68108, and bovine serum gamma globulin (BGG), Armour and Co., Lot No. C 904, were labeled with $I^{131}$ (I*) as previously described(10).

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† Present address: Dept. of Medicine, Northwestern Univ. Med. School.

‡ Senior Research Fellow of the Nat. Inst. of Health.

*Antisera.* Rabbit antisera against BSA and BGG were obtained from hyperimmunized rabbits prepared by a series of subcutaneous and intravenous injections of either BSA or BGG, totaling 250-300 mg, given over a 2-4 month period(11). The last injection was given 7 days before bleeding.

Antibody levels of the antisera were determined by the quantitative immunochemical procedures developed by Heidelberger and co-workers(11). Total N in antigen-antibody precipitates and in the various protein solutions was measured by the Markham modification of the micro Kjeldahl technic (12). The pooled anti BSA serum contained 2.18 mg of antibody N/ml, and the pooled anti BGG serum contained 1.20 mg antibody N/ml.

*Elimination of I* antigen from rabbits.* Albino rabbits weighing 2.5-3 kg were passively immunized by intravenous injection of 1.1-45.0 $\mu$g rabbit anti BSA N or rabbit anti BGG N and 24 hours later injected intravenously with 0.1-4 $\mu$g I* BSA N or I* BGG N. Control rabbits received comparable amounts of I* antigen but no antisera. Also, non-immunized rabbits received 1-10 mg unlabeled antigen immediately prior to administration of I* antigen in order to determine if a portion of the I* antigens was eliminated by natural globulin capable of acting as antibody. If a small amount of the antigen could be eliminated by natural globulin, the blood of rabbits receiving the prior injection of unlabeled antigen should have contained more I* antigen than the rabbits not receiving a prior injection of unlabeled antigen. Rabbits were bled from ear veins at intervals, and radioactivity of the trichloroacetic acid precipitable fraction of the serum was determined in a NaI crystal scintillation counter and recorded as per cent of total injected protein bound radioactivity remaining in the total blood volume. Sufficient counts were obtained to insure a maximum counting error of no greater than 5%.

One group of rabbits was injected intravenously with a mixture of I* BSA-anti BSA complexes prepared at equivalence with 1 $\mu$g antibody N and .18 $\mu$g antigen N and incubated at 37°C for 30 minutes prior to injection. Circulating I* BSA-anti BSA complexes were determined as previously described(13), a modification of the method of Farr(14). One ml of 0.15 M NaCl was added to 1.0 ml of serum and mixed, and 2 ml of saturated ammonium sulfate (pH 7.2-7.5) were added to the mixture. After 30 minutes at 0-1°C, the mixture was centrifuged and the supernatant decanted. After 2 washings with 50% saturated ammonium sulfate the precipitate was counted in a NaI crystal scintillation counter. *Elimination of I* antigen-antibody complexes from guinea pigs.* Guinea pigs weighing approximately 500 g were injected intravenously in the lateral penile veins with a mixture of either I* BSA-rabbit anti BSA or I* BGG-rabbit anti BGG prepared *in vitro* in 5% Dextrose in water. Complexes containing .03 to 3 $\mu$g antibody N/ml were prepared using weight ratios of 1 $\mu$g of BSA N to 5.5 $\mu$g of anti BSA N and 1 $\mu$g of BGG N to 3 $\mu$g of anti BGG N and injected in a total volume of 1 ml. Control animals were injected with comparable amounts of I* antigen but no antiserum. Radioactivity of each animal was determined by counting the whole animal in a large well-type geiger counter immediately after injection and at various intervals. *Elimination of I* antigen-antibody complexes from mice.* Albino mice weighing 20-25 g were treated the same as guinea pigs except that the I* antigen-antibody complexes and I* antigen were injected into the dorsal tail veins. Total volume injected was 1 ml.

All animals used in this study received KI in their drinking water during, and for several days prior to, the experiment.

*Results. Elimination of I* antigen in sensitized and normal rabbits.* Elimination of I* BSA and I* BGG from both normal rabbits and rabbits passively sensitized with 1.1-45.0 $\mu$g of rabbit antibody N is shown in Table I. In the rabbits injected with antibody, the antigen was almost completely eliminated from the blood in 24 hr, while in the control rabbits, 22-34% of the antigen still remained at this time. Ten minutes after injection of I* antigen into rabbits sensitized with 1.1 and 4.5 $\mu$g of antibody N, the I* antigen re-

TABLE I. Elimination of I* Antigen from Normal and Passively Immunized Rabbits.†

| Antibody N inj., μg | Antigen N inj., μg | % of total inj. I* antigen remaining in blood | | | | % of total inj. I* antigen bound to globulin | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 10 min. | 1 hr | 6 hr | 24 hr | 10 min. | 1 hr | 6 hr | 24 hr |
| 45 anti BSA | 4 I* BSA | 47 | 13 | 2 | 0 | 46 | 11 | 1 | 0 |
| None | *Idem* | 86 | 67 | 44 | 25 | 3 | 2 | 1 | 0 |
| " | 1,500 BSA prior to 4 I* BSA | 89 | 72 | 44 | 24 | 3 | 2 | 1 | 0 |
| 45 anti BGG | 4 I* BGG | 13 | 10 | 1 | 0 | | | | |
| None | *Idem* | 93 | 82 | 51 | 28 | | | | |
| " | 1,500 BGG prior to 4 I* BGG | 87 | 78 | 49 | 26 | | | | |
| 22.5 anti BSA | 2 I* BSA | 36 | 8 | | 1 | 37 | 6 | | |
| None | *Idem* | 98 | 79 | | 28 | 1 | 1 | | |
| " | 750 BSA prior to 2 I* BSA | 88 | 75 | | 30 | 1 | 1 | | |
| 22.5 anti BGG | 2 I* BGG | 33 | 6 | | 0 | | | | |
| None | *Idem* | 89 | 72 | | 32 | | | | |
| " | 750 BGG prior to 2 I* BGG | 85 | 68 | | 29 | | | | |
| 4.5 anti BSA | .4 I* BSA | 88 | 43 | | 1 | 90 | 42 | | |
| None | *Idem* | 98 | 78 | | 30 | 1 | 1 | | |
| " | 150 BSA prior to .4 I* BSA | 98 | 76 | | 26 | 2 | 1 | | |
| 4.5 anti BGG | .4 I* BGG | 73 | 22 | | 2 | | | | |
| None | *Idem* | 94 | 75 | | 30 | | | | |
| " | 150 BGG prior to .4 I* BGG | 75 | 64 | | 26 | | | | |
| 1.1 anti BSA | .1 I* BSA | 93 | | | 1 | | | | |
| None | *Idem* | 97 | | | 27 | | | | |

† Recorded as avg of results from 2 rabbits.

maining in the blood was considerably higher than in rabbits sensitized with the larger amounts of antibody. In the groups sensitized with 4.5, 22.5 and 45 μg of anti BSA N, almost all of the I* BSA remaining in the blood at the 10 minute bleeding was present in the form of antigen-antibody complexes.

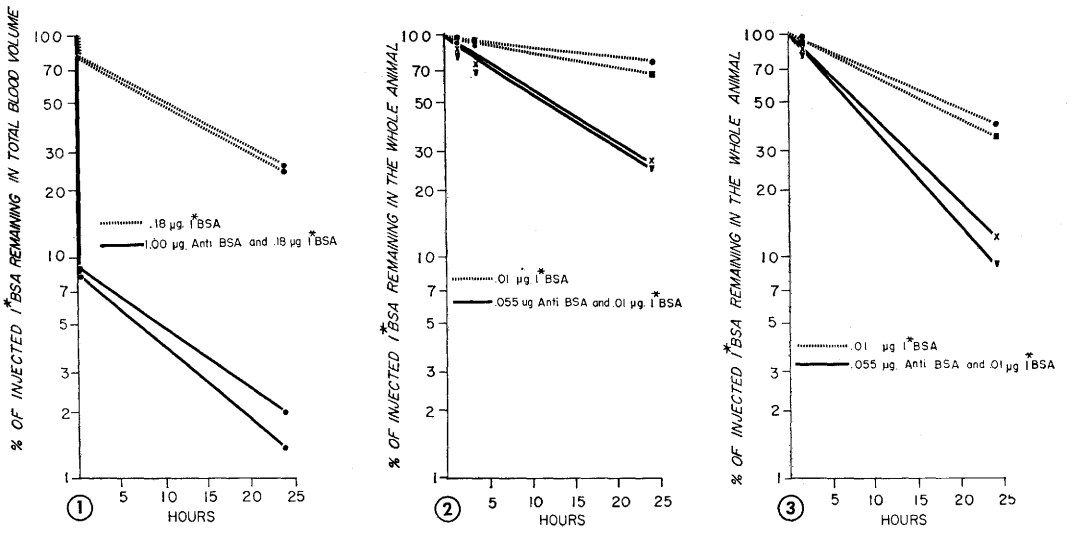
To determine if a portion of the I* antigens was eliminated by natural globulin capable of acting as antibody, 10 mg of unlabeled homologous antigen was injected in some of the normal rabbits prior to injection of the I* antigen. If a small amount of globulin in the serum of immunized animals was capable of acting as a natural antibody, the blood of rabbits receiving the prior injection of unlabeled antigen should contain more I* antigen than the blood of rabbits not receiving a prior injection of excess unlabeled antigen. However, there was no significant difference in level of I* antigen during a 24 hour period in the blood of rabbits receiving the prior injection of antigen and in those not receiving prior antigen (Table I), nor was a significant difference in amount of antigen-antibody complexes detected between the sera of the two groups.

The rapid elimination of I* antigen-antibody complexes prepared at equivalence with 1 μg of anti BSA N is shown in Table II and Fig. 1. As evidenced by the level of I* in the sera, these complexes were almost com-

TABLE II. Elimination of Preformed I* BSA-anti BSA Complexes from Blood of Rabbits.†

| Anti BSA N inj., μg‡ | I* BSA N inj., μg | % of total inj. antigen remaining in blood | |
|---|---|---|---|
| | | 10 min. | 24 hr |
| 1 | .18 | 8.5 | 1.5 |
| None | .18 | 86 | 24.5 |

† Recorded as avg of results from 2 rabbits.
‡ Anti BSA combined with I* BSA during 30 min. incubation period *in vitro*.




Elimination of I* BSA from animals inj. with I* BSA-anti BSA complexes or I* BSA. Fig. 1, rabbits. Fig. 2, guinea pigs. Fig. 3, mice.

pletely eliminated 10 minutes following injection.

*Elimination of I* antigen-antibody complexes from guinea pigs and mice.* Elimination of radioactivity from guinea pigs and mice injected intravenously with I* antigen-antibody complexes prepared *in vitro* at equivalence and from guinea pigs and mice injected with I* antigen alone is shown in Tables III and IV and in Fig. 2 and 3. Animals which received I* BSA-anti BSA complexes or I* BGG-anti BGG complexes showed a significantly more rapid decrease in total body radioactivity at 24 hours than animals which received I* BSA or I* BGG

TABLE III. Elimination of Both Free I* Antigen and I* Antigen-Antibody Complexes from Guinea Pigs.†

| Antibody N inj., µg | Antigen N inj., µg | % of total inj. antigen remaining in whole animal 2 hr | 5 hr | 16 hr | 24 hr |
|---|---|---|---|---|---|
| 3 anti BGG | 1 I* BGG | 78 | 66 | 31 | 18 |
| None | *Idem* | 93 | 84 | 66 | 55 |
| 1.1 anti BSA | .2 I* BSA | 86 | 67 | 26 | 14 |
| None | *Idem* | 96 | 88 | 64 | 54 |
| .3 anti BGG | .1 I* BGG | 94 | 68 | 32 | 16 |
| None | *Idem* | 93 | 85 | 64 | 53 |
| .03 anti BGG | .01 I* BGG | 78 | 69 | | 18 |
| None | *Idem* | 98 | 92 | | 49 |
| .055 anti BSA | .01 I* BSA | 85 | 72 | | 26 |
| None | *Idem* | 96 | 94 | | 75 |

† Recorded as avg of results from 2 animals.

TABLE IV. Elimination of Both Free I* Antigen and I* Antigen-Antibody Complexes from Mice.†

| Antibody N inj., µg | Antigen N inj., µg | % of total inj. antigen remaining in whole animal 2 hr | 5 hr | 16 hr | 24 hr |
|---|---|---|---|---|---|
| 3 anti BGG | 1 I* BGG | 97 | 95 | 21 | 6 |
| None | *Idem* | 97 | 88 | 54 | 38 |
| .3 anti BGG | .1 I* BGG | 83 | 56 | 19 | 7 |
| None | *Idem* | 91 | 85 | 58 | 39 |
| .055 anti BSA | .01 I* BSA | 84 | | | 10 |
| *Idem* | .025 I* " | 86 | | | 22 |
| " | .05 I* " | 90 | | | 25 |
| " | .1 I* " | 93 | | | 31 |
| None | .01 I* " | 94 | | | 36 |

† Recorded as avg of results from 2 animals.

alone. Mice injected with I* BSA-anti BSA complexes prepared with 2.5, 5 and 10 times the amount of antigen necessary to precipitate all of the antibody at equivalence (2.5, 5 and 10 times equivalence) showed progressive decrease in rate of immune elimination of the I* BSA with increasing degree of antigen excess (Table IV). The mice receiving I* BSA-anti BSA complexes prepared with I* BSA at 10 times equivalence demonstrated rates of antigen elimination which approached rates of elimination in control mice which were injected with I* BSA alone.

*Discussion.* Our results show that immune elimination of I* antigen can be used to detect the presence of trace amounts of anti-

body in passively immunized animals, animals injected with I* antigen-antibody complexes and, presumably, actively sensitized animals. The results were shown in passively immunized rabbits or in rabbits, mice and guinea pigs injected with I* antigen-antibody complexes. Rapid elimination of an appropriate amount of I* antigen resulted from passive sensitization of a rabbit with 1 $\mu$g of antibody N. In a 3 kg rabbit with a total plasma pool equal to 300 ml, amount of antibody N per ml of plasma detected was .0033 $\mu$g. Therefore, immune elimination may be detected in passively (and presumably actively) sensitized rabbits in which the antibody levels are as low as .0033 $\mu$g antibody N/ml of plasma.

The I* antigen-antibody complexes, containing a total of no more than .03 $\mu$g of antibody, were rapidly eliminated from guinea pigs as shown by more rapid decrease in whole body radioactivity. Thus, in a 500 g animal with a total plasma volume of 25 ml, .0012 $\mu$g of antibody N/ml of plasma should be detectable in a sensitized guinea pig.

The amounts of antibody used for passive immunization and for injection of I* antigen-antibody complexes were injected in a volume of 1 ml. Therefore, the sensitivity of the method can be expressed as $\mu$g of antibody N/ml of serum and was found to be from .0012-.0033 $\mu$g antibody N. However, if the same amount of antibody were present in a larger volume which is limited only by the amount which can be safely injected intravenously in a given species of animal, approximately 20 ml per rabbit or 5 ml per guinea pig, the relative sensitivity of the test as expressed by $\mu$g antibody N/ml of serum would be greatly increased.

The sensitivity of this method compares favorably with that of other methods of detecting antibody(15). For the quantitative precipitin reaction, the lower limits of detection are approximately 20 $\mu$g of antibody N.; for the hemagglutination reaction, .003 $\mu$g of antibody N/ml serum; for local passive cutaneous anaphylaxis, .03 $\mu$g antibody N/ml serum; for the complement fixation reaction, .1 $\mu$g antibody N/ml serum; and for passive anaphylaxis in the guinea pig, 10 $\mu$g of antibody N. The precipitation of I* antigen-antibody complexes by ammonium sulfate precipitation(14) is a method equally sensitive to that observed here, but this technic is limited to systems in which the free antigen is not precipitated by the method used to precipitate the antibody or the antigen-antibody complexes.

The rapid immune elimination technic cannot be used to make an absolute quantitation of antibody. However, the results in mice injected with antigen-antibody complexes prepared at varying antigen-antibody ratios indicate that the use of varying amounts of I* antigen could be employed to approximate the amount of antibody (Table IV). The maximal amount of I* antigen which could be injected and still be eliminated at an increased rate approximated the amount which would combine with the total circulating antibody at equivalence.

The level of antibody which can be detected by the immune elimination technic is limited by the smallest amount of I* antigen that can be traced *in vivo,* which is determined by the specific activity of the antigen. Specific activity of the antigen is limited by the amount of I* which can be conjugated to protein without chemical or radiochemical denaturation of protein. In our experience, using commercially available carrier-free I*, the limit of specific activity is approximately 2-3 $\mu$c per $\mu$g protein N.

Rabbits injected with 1.1 to 4.5 $\mu$g of antibody N and 1/10 of this amount of I* antigen retained considerably more I* antigen in their circulation after 10 minutes than rabbits injected with 22.5 and 45 $\mu$g of antibody N and proportionately the same amounts of I* antigen. Since in all anti BSA immunized animals tested, the I* antigen present in the blood was almost entirely in the form of antigen-antibody complexes after 10 minutes, it seems likely that the rabbits injected with the smaller amounts (1.1-4.5 $\mu$g) of antibody and antigen contained complexes which were of different composition than the antigen-antibody complexes formed in rabbits injected with the larger amounts (22.5-45 $\mu$g). Antigen-antibody complexes formed in rabbits injected with 1.1 and 4.5 $\mu$g of antibody N were

probably diluted in the plasma to such a degree that they were smaller and removed more slowly by phagocytic cells than complexes formed by higher concentrations of antigen and antibody.

Elimination of antigens from rabbits passively immunized with 1.1-45.0 $\mu$g of antibody N bears on the natural selection theories of antibody formation. These theories postulate that the normal gamma globulins consist of a variety of molecular species(16), one or more of which by chance are likely to react with any given antigen. The effect of 1.1 $\mu$g of anti BSA per rabbit on elimination of I* BSA as compared with controls injected with I* BSA alone indicates that any natural anti BSA globulins must be present in amounts less than the 1.1 $\mu$g of antibody N per rabbit which was used for passive immunization. In addition, unsensitized normal rabbits failed to show evidence of a significant amount of circulating I* BSA-anti BSA complexes following injection of small amounts of I* BSA (Table I). Although a trace amount of I* BSA was precipitated with globulin 10 minutes following injection of I* BSA, this was apparently non-specific since a prior injection of a large amount of non-labeled BSA failed to decrease the amount of I* BSA precipitated with the globulins. If a 3 kg rabbit has approximately 3 g of gamma globulin, 1.1 $\mu$g of anti BSA N would be less than 1/400,000 of the rabbit's total gamma globulin. Thus, if natural globulins capable of combining with the various BSA antigenic determinants exist, they must total less than this fraction of the gamma globulin. Considering the .5 kg guinea pigs injected with antigen-antibody complexes containing .03 anti BGG N, this amount of antibody was equal to approximately 1/2,555,000 of the gamma globulin in the animal. Since this amount of antibody caused an immune elimination, any natural globulins capable of combining with the various BGG antigenic determinants would necessarily be present in an amount smaller than $2 \times 10^{-7}$ g of antibody protein per animal. The levels of naturally occurring antibody against these serum protein antigens are considerably lower in the rabbit and guinea pig than would be expected if the gamma globulin were made up of a random mixture of 5,000(16) to 50,000(17) different molecular species. Therefore, if the gamma globulin of these species consists of mixtures of some such number of molecular species, the amounts of these different molecules must be extremely unequal.

*Summary.* Immune elimination of antigen using very small amounts of antigen and antibody occurs in characteristic fashion in rabbits, guinea pigs and mice and may be used as an additional sensitive method for demonstration of small amounts of antibody against serum protein antigens. Consideration of these findings in regard to the hypothesis of natural antibodies is discussed.

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