Effects of Rheumatoid Factor on *in vivo* Distribution of Aggregated Human IgG and Antigen-Antibody Complexes. (32201)

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Previous studies have shown that rheumatoid factor (RF) can inhibit the serum complement lowering effect of intraperitoneally injected aggregated human IgG (HAggIgG) in the rat(1). Further studies also revealed an inhibitory role for RF when HAggIgG was injected with RF intrapleurally and indeed, though to a lesser extent, when injected intraarticularly in exercised rats(2). Such an effect was *not* noted, however, when the IgG and RF were injected together intravenously, even when these substances had been previously incubated together *in vitro*(1).

The current studies were undertaken to determine more definitively the mechanism of action of RF in modifying the fate of HAggIgG injected intraperitoneally and, in addition, to study the effects of RF on an antigen-antibody complex under similar conditions. The major approach was to study the distribution of globulins labelled with I^{131} three hours after injection.

Materials and methods. Rheumatoid euglobulin was prepared from pools of human sera containing high titers of RF activity. The euglobulin precipitate was redissolved in a minimal volume of saline and then dialysed for one hour in 0.1 M acetate buffer, pH 4.1. Subsequently the solution was eluted from a Sephadex G-200 column in acetate buffer, and the IgM peak concentrated to original serum volume with sucrose and dialysed in 0.1 M glycine-saline buffer, pH 8.2. RF-negative serum was processed in the same manner. One RF-positive macroglobulin solution was exhaustively absorbed with insoluble HAggIgG to prepare another type of RF-negative control material.

Human FII was obtained from Mann Research Laboratories and aggregates of IgG prepared as previously reported(1), except that 3 reprecipitations of the aggregates were always employed to remove as much native IgG as possible. The aggregates thus produced were found to sediment at between 30S and 40S on analysis in the Spinco Model E Ultracentrifuge and were essentially free of 7S material. After dialysis in 0.15 M phosphate buffer, pH 8.2, a 0.5 to 1.0 ml solution containing about 10 mg of protein was iodinated with about 300 microcuries of carrier-free NaI^{131}†(3) and immediately passed through a column of DeAcidite FF, previously equilibrated with saline. Tubes of eluate containing peak activity were pooled and protein determined by a "micro" modification of the method of Lowry *et al*(4). Labelled aggregates (HAggIgGI*) were subsequently reacted with control-IgM or RF-IgM (derived from 1 to 2 ml of sera and brought to equal protein concentrations) and immediately injected intraperitoneally in a final volume of 3.0 ml.

Purified human thyroglobulin(5), labelled with I^{131}(3), and subsequently purified on Sephadex G-200 to remove free I^{131} as well as fragments of thyroglobulin, was reacted with a pool of sera obtained from patients with autoimmune thyroiditis‡ and which had been shown to contain antithyroglobulin antibody both by immunodiffusion and tanned sheep red blood cell agglutination techniques. Though no precipitating antibody could be demonstrated in standard precipitin tests holding antibody constant and adding varying amounts of antigen, a precipitin curve was easily obtained when a constant amount of RF-IgM material was added to the tubes. An amount of thyroglobulin was then selected

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‡ Kindly sent to us by Drs. Ivan Roitt and J. G. Feinberg.

for the *in vivo* tests which gave maximum precipitation in this system, as well as an amount in greater antigen excess. All sera were first heat-inactivated and made essentially lipid-free by centrifugation at 35,000 RPM for 1 hour at 4°C. For the *in vivo* tests, labelled thyroglobulin (TgI*) and antithyroglobulin sera (AntiTg) were first reacted at 37°C for one hour and then left overnight at 4°C. Constant amounts of control and RF-IgM were then added to appropriate tubes one hour before injection into rats, and incubated at 37°C.

Rats were Wistar males weighing about 250 g. Blood was obtained either from the tail or by cardiac puncture, and organs were removed after exsanguination under ether anesthesia to obtain specimens as free of blood as possible. Radioactivity of blood and organs was monitored in a well-type scintillation counter (SELO, Milano, Italia) and counts were corrected for background activity and adjusted for variations in organ weights (counts/sec/g). Serum was further fractionated by density gradient centrifugation in sucrose in a Spinco Model L centrifuge using a SW39 head at 35,000 RPM for 15 hours at 4°C. Aliquots obtained through a hole off-center in the bottom of the tubes were assayed for radioactivity.

Complement fixation tests were carried out by the method of Kabat and Mayer(6).

*Results. HAggIgGI** in vivo. In one experiment 4 rats divided into 2 groups received 0.5 mg of HAggIgGI* each, plus either control or RF-IgM. Three hours after intraperitoneal injection, animals were exsanguinated by cardiac puncture and lungs, kidneys, heart, spleen, and a liver slice were removed for weighing and counting. Fig. 1 shows the distribution of counts in the various organs and sera averaged for these two groups. It is apparent that there are less than half as many counts in the sera of animals given aggregates plus RF-IgM as in sera of animals given aggregates plus control-IgM. There is a similar marked difference in total counts in both the liver and spleen. Counts in the lungs, kidneys, and heart, however, showed no apparent differences. Counts in all organs were repeated

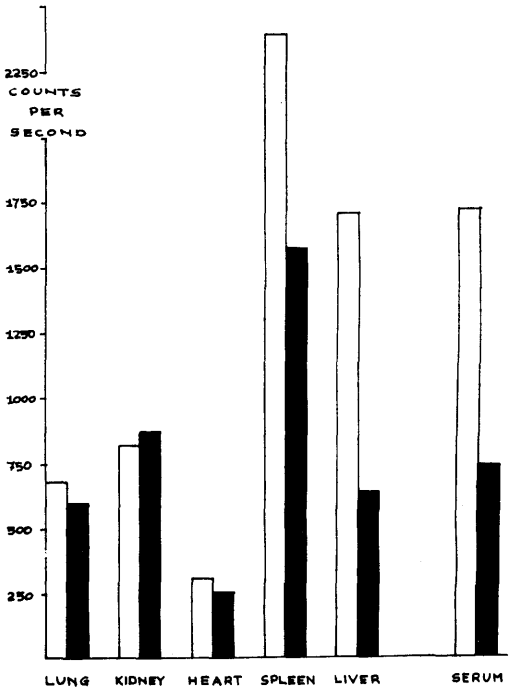

FIG. 1. Distribution of radioactivity in organs and sera of rats injected 3 hours previously with labelled aggregated human IgG plus either control-IgM (clear bars) or RF-IgM (black bars).

after several washings with saline, and though all counts fell thereafter, they remained proportional, and thus did not appear to alter the interpretation of the results.

An 0.25 ml aliquot of serum from each rat was diluted to 0.5 ml in saline containing bromphenol blue and subjected to sucrose gradient centrifugation. Results in 2 rat sera are shown in Fig. 2 where the sample assays are reported as per cent of total amounts. It can be seen that 34% of the activity of the uninjected HAggIgGI* resided in the pellet, while another 40% was found in a rapidly sedimenting fraction (about 20S). Very little activity was found in the upper part of the gradient. Radioactivity from the serum of an animal receiving HAggIgGI* plus control-IgM showed 21.5% activity in the pellet, somewhat less in the 20S range, and proportionally more in the upper portions of the tube. On the other hand, the animal which additionally received RF-IgM, showed only 11% activity in the pellet, similar activity to the control sera in the lower part

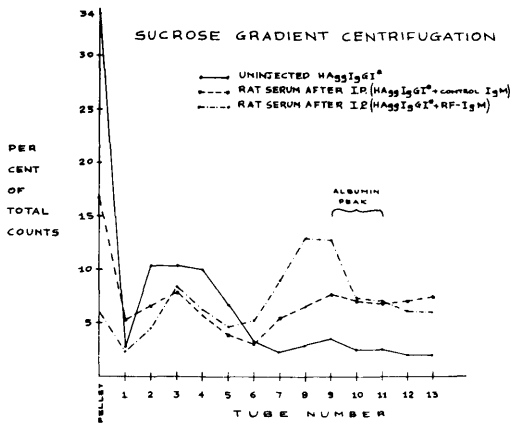

FIG. 2. Distribution of radioactivity in a sucrose gradient comparing uninjected HAggIgGI* with post-injection sera from animals receiving HAggIgGI* preparations three hours previously.

of the gradient, and much higher percentage activity in the slower sedimenting area. Results for the other two rats were essentially as these. Furthermore, several other experiments using slight variations in technique gave similar overall results.

AntiTg and role of RF. AntiTg was demonstrated in a pool of sera as noted under *Methods*. In double diffusion studies, 2 fine white lines were noted as well as a pronounced "clear line." The tanned sheep red blood cell test revealed a titer of agglutinating antibody to 1:250,000,000. Experiments to discover the equivalence point were at first unsuccessful, as no precipitate could be discovered in standard assay tubes even after one week in the cold. Since previous work has shown that antiTg antibodies are almost exclusively IgG(7), RF-IgM was added to each tube along with antigen and antibody to determine if any reaction might be noted. Control-IgM was added to a similar series. Results of one assay are shown in Table I. It is apparent that gross precipitation was noted only in tubes containing RF-IgM, and that no precipitation was noted in the absence of Tg or with IgM from a normal individual. Other experiments, not shown, using this same RF-IgM material, which was then absorbed with insoluble HAggIgG, also failed to give a precipitate. It was tentatively concluded that the factor responsible for the precipitation was indeed IgM-RF.

Tg-antiTg complexes were tested for complement fixing ability with both human and guinea pig sera as a source of complement, but very little, if any, fixation was noted. This was in confirmation of the previous findings of Roitt *et al*(8).

TgI-antiTg complexes* in vivo. 5 mg of purified human Tg was labelled with about 500 microcuries of NaI^{131} and tubes with peak radioactivity pooled after passage through a column of Sephadex G-200. Two ratios of TgI*-antiTg were selected, based on the previous experiments using RF-IgM to determine the equivalence point; one at equivalence and one in moderate antigen excess. All animals were to receive 0.1 ml of antiserum, plus either 0.2 mg or 0.3 mg of TgI.* These groups were in turn divided into two groups so as to receive, in addition, either control or RF-IgM.

TABLE I. Precipitation After Incubation of Antithyroglobulin Sera with Thyroglobulin and Human IgM for 1 Hr at 37°C and Overnight at 4°C.

Pooled autoimmune thyroiditis sera (ml)*	Human thyroglobulin (mg)	IgM control (ml)*	IgM rheum. (ml)*	Visible ppt†	O.D. at 280 mu of digested precipitate‡
.1	0	.5	—	—	.014
.1	.2	.5	—	—	.004
.1	.3	.5	—	—	.010
.1	.5	.5	—	—	.000
.1	0	—	.5	—	.004
.1	.2	—	.5	+++	.161
.1	.3	—	.5	++	.075
.1	.5	—	.5	—	.000

* Heat inactivated and clarified of lipid.
† Total reaction volume was 1.5 ml.
‡ Final volume was 2.5 ml of 1.0 N NaOH.

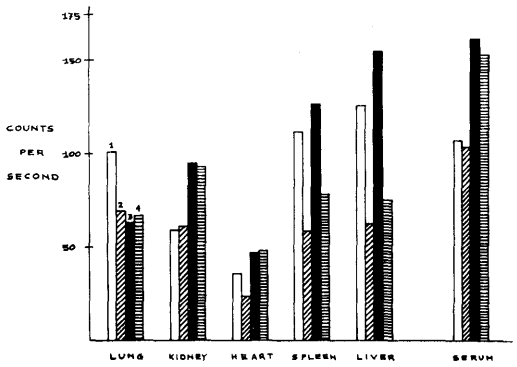

FIG. 3. Distribution of radioactivity in organs and serum of rats injected 3 hr previously with labelled thyroglobulin (TgI*)-antiTg complexes, in 2 different ratios, plus either control-IgM or RF-IgM. Bar 1. TgI*-antiTg (at equivalence) + control-IgM; Bar 2. TgI*-antiTg (at equivalence) + RF-IgM; Bar 3. TgI*-antiTg (antigen excess) + control-IgM; Bar 4. TgI*-antiTg (antigen excess) + RF-IgM.

Three hours after intraperitoneal injection all animals were exsanguinated and organs recovered. Fig. 3 shows the results for the 4 groups. In this experiment there was no marked difference between the animals receiving control or RF-IgM in assays of serum, lungs, kidneys, and heart, though there was a slight difference in lung counts in animals receiving TgI*-antiTg complexes at equivalence. As expected, all 4 animals receiving 0.3 mg of TgI* showed generally higher counts in all tissues (except the lungs) than animals receiving only 0.2 mg of TgI.* In the spleen and liver, however, a marked difference between RF-IgM and control-IgM treated animals was noted in all cases. As in experiments with the aggregates, much less activity was found in the spleen and liver of animals also receiving RF-IgM.

Sera from one control and one RF treated animal (antigen excess group) were subjected to sucrose gradient centrifugation. as was a sample of TgI*-antiTg-control IgM material which had not been injected. The results are shown in Fig. 4. The large amount of material in the pellet of the tube containing uninjected material is striking in contrast to the tubes containing serum from injected animals. No obvious differences are noted in the distribution of radioactivity in the 2 test sera, though there again appears to be less activity in the pellet of the tube containing sera from the animal injected with complexes plus RF-IgM. Almost all the activity in these 2 sera resides in the upper part of the gradient, suggesting extensive breakdown of the thyroglobulin, either with control or RF-IgM material.

Discussion. These studies essentially confirm and extend previous findings on a metabolic effect of RF(1). While the earlier work showed that RF could inhibit the anticipated fall in serum complement levels following the intraperitoneal injection of HAggIgG, no information was available to explain the precise mechanism. Data presented here strongly suggest that 1) RF inhibits the absorption of aggregates from the peritoneum by whatever route and 2) at the same time promotes breakdown of the aggregates, presumably by phagocytic cells. The former point is supported by the finding, 3 hours post-injection, of a lower total amount of radioactivity in the serum, spleen, and liver of animals given aggregates plus RF-IgM, than in animals given aggregates plus control-IgM. The second point is supported by the finding in serum of a relatively smaller per-

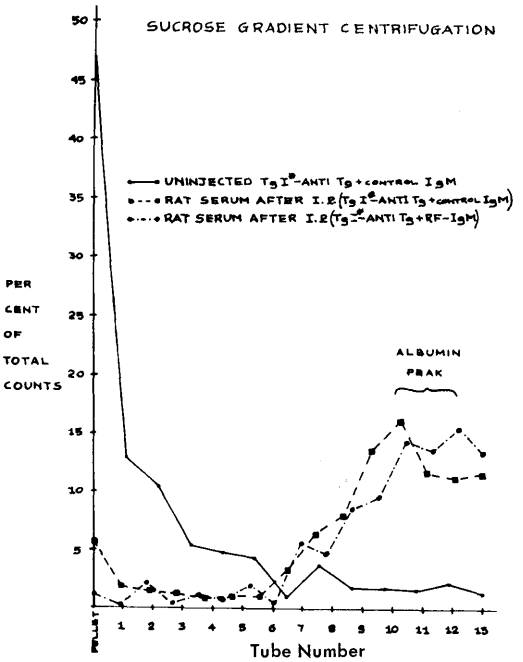

FIG. 4. Distribution of radioactivity in a sucrose gradient comparing uninjected TgI* material with post-injection sera from animals receiving TgI* preparations 3 hours previously.

centage of large radioactive aggregates, and, obviously, a greater percentage of smaller aggregates in animals given IgM-RF as opposed to controls. Thus there is evidence for both a quantitative and a qualitative effect of RF on HAggIgG metabolism in the rat peritoneum.

Studies with a human antibody-antigen complex proved somewhat more surprising and difficult to interpret. Of most interest was the marked reactivity of the system with RF-IgM *in vitro,* while at the same time showing only very poor, if any, reactivity with either human or guinea pig complement. These findings suggest that the binding sites for RF and complement on the IgG molecules may indeed be different, a finding in keeping with the report of Wiedermann *et al*(9). Whether this observation is valid for other non-complement fixing systems remains to be seen. The possibility of using RF-IgM as a tool in the quantitative precipitin test for human antibody also deserves further exploration.

In vivo it appeared that RF-IgM could also inhibit absorption of human antibody-antigen complexes from the peritoneum in view of the lower amount of radioactivity found in the liver and spleen of animals additionally given RF-IgM. That this finding was not reflected in the serum was surprising, but in view of the large amount of breakdown of the thyroglobulin, as shown by sucrose density centrifugation, it may simply reflect the large number of fragments in serum which are *not* sequestered in the liver and spleen. It will be of great interest to explore other human antibody-antigen systems to determine their fate under similar and varying circumstances.

Summary. HAggIgG and Tg-antiTg complexes were labelled with I^{131} and injected into the peritoneum of rats. These rats were also injected IP with human IgM material, either containing, or free from, RF activity. Animals receiving aggregates plus RF-IgM showed much less radioactivity in the serum, liver, and spleen than controls, 3 hours after injection. Similar findings were noted in the complex-treated animals, except that no difference was noted in serum and evidence was obtained for extensive and rapid thyroglobulin breakdown. In aggregate-treated animals, qualitative differences were also noted in the serum on sucrose gradient centrifugation, suggesting another effect of RF-IgM on aggregate metabolism. Finally it was noted *in vitro* that extensive RF binding could occur in the Tg-antiTg system, a system which at the same time fixed complement very poorly, if at all.

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