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THE EFFECT OF COMPLEMENT-DEPLETION OR MANNOSYL RECEPTOR BLOCKADE ON THE *IN VIVO* CLEARANCE OF ANTIGEN DURING A PRIMARY IMMUNE RESPONSE IN RABBITS

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Summary.—The effect of complement-depletion mediated by cobra venom factor or mannosyl receptor blockade induced by ovalbumin injections on the blood clearance of *in vivo*-generated soluble immune complexes were analysed in 2 variants of a primary immune elimination model system in rabbits. In the first variant, animals were immunized with particulate antigen to elicit high titre IgM antibodies, and received radiolabelled antigen on the first day of immunization. In the second variant, radiolabelled antigen was introduced on Day 8 of the immunization schedule.

The results showed that (a) immune clearance of antigen (Days 6–8) was not inhibited by prior decomplementation of the animals with cobra venom factor, and (b) injection of ovalbumin just prior to immune clearance did not significantly alter the clearance kinetics of the resulting immune complexes.

Thus, the results indicate that clearance of soluble immune complexes generated during a primary immune response is not mediated *via* C3b–C3b receptor interaction or *via* mannosyl–mannosyl receptor interaction in the reticuloendothelial system, but proceeds through other mechanisms perhaps involving Fc receptors.

THE PRECISE physiological mechanisms involved in clearing soluble immune complexes (IC) from the circulation are not well understood. In previous reports, we have studied the effects of complement-depletion on the clearance of either preformed IC containing IgM antibodies (IgM.IC) in predetermined antigen/antibody ratios (Harkiss and Brown, 1981), or IgM.IC generated *in vivo* following injection of a pulse of IgM antibody into rabbits with circulating antigen (Brown and Harkiss, 1981). In the first model, the antigen/antibody ratio was fixed before injection, while in the second the ratio varied continuously in the direction of antigen excess. No significant inhibition of IgM.IC clearance was found in either model when the animals were depleted of complement by cobra venom factor (CVF).

In both cases, however, the physiological conditions described are unlikely to occur in the normal clearance of antigen or in the evolution of human disease. For this reason, it was considered necessary to develop a more natural model system, which enabled the clearance mechanisms for IgM.IC to function under normal physiological conditions.

The model system chosen consisted of monitoring the clearance of IC formed during the primary IgM response to a particulate antigen. The rapid removal of the latter enabled small physiological amounts of antigen to be introduced into the circulation, and its clearance followed after development of the IgM immune response. Two variants of the model were studied. In the first, the radiolabelled antigen was injected on the first day of immunization, and its natural catabolic and immune clearance followed over the

next 10 days (primary clearance model). In the second variant the animals were immunized as usual, but the radiolabelled antigen was injected on Day 8, and its clearance monitored over a period of 1 h (Day 8 clearance model).

The role of complement and mannosyl receptors in these clearance models was examined by depleting rabbits of C3 with CVF, or examining the inhibitory effects of pre-injecting animals with ovalbumin, respectively.

METHODS

Animals and reagents.—Male New Zealand White rabbits, 2–3 months old and weighing about 3 kg were used throughout. Each experimental group consisted of 3 rabbits. Bovine serum albumin (BSA), ovalbumin, dithiothreitol (DTT) and iodoacetamide (IA) were obtained from Sigma. Purified CVF was kindly supplied by Professor P. J. Lachmann, MRC Centre, Cambridge.

Immunization and radiolabelling.—High-titre IgM antibodies were raised by giving rabbits 2 i.v. injections spaced 2 days apart of 1 ml of 50% BSA-linked sheep erythrocytes prepared as described previously (Harkiss and Brown, 1981). BSA and ovalbumin were radiolabelled with ^{125}I and ^{131}I respectively by the chloramine T method. The labelled proteins were exhaustively dialysed against saline before injection into rabbits. Specific activities for different preparations averaged about 20×10^3 counts/100 sec/μg BSA.

Injection of ^{125}I-BSA, CVF and ovalbumin.—All injections were given *via*, and blood samples taken from, the marginal ear veins. In the primary clearance model, 1 mg of ^{125}I-BSA was injected in 1 ml of saline into rabbits and its clearance followed by taking 1 ml blood samples at various time intervals. In the Day 8 clearance model, 0·5 mg of ^{125}I-BSA was injected. In decomplementation experiments, CVF was injected i.v. 24 h before initiation of ^{125}I-BSA clearance. C3 values were reduced to almost undectable levels for 5–6 days by this treatment.

To determine the effect of mannosyl groups on the clearance of ^{125}I-BSA/anti-BSA IC, each rabbit received 2·1 g of ^{131}I-ovalbumin in 8 ml of saline 4 days after injection of BSA in the primary clearance model. This amount was calculated to produce a concentration of 1–2 mg/ml of blood after 24 h, and slightly lower values during the primary clearance phase of ^{125}I-BSA. In the Day 8 clearance model, each animal received approximately 0·2 g of ovalbumin in 3 ml of saline 5 min before injection of ^{125}I-BSA. All rabbits remained healthy and alert following each injection and remained so throughout the period of observation. 100 μl of each serum sample obtained was counted for radioactivity in a gamma counter set to discriminate optimally the 2 photopeaks. The specific activity of each labelled protein was chosen to produce a 10-fold excess of ^{125}I over ^{131}I counts, so that correction for carryover from the ^{131}I to ^{125}I channel was unnecessary.

Antibodies to BSA.—Anti-BSA antibodies in rabbit sera were measured as described previously (Harkiss and Brown, 1981). To assess the extent of contamination with IgG antibodies, the serum samples were reduced in 10 mM DTT in 0·2M Tris-HCl buffer pH 8 for 30 min at 37°, then alkylated in 30 mM IA in the same buffer in the dark for 15 min at room temperature. The samples were then re-assayed immediately for anti-BSA antibody activity without further treatment.

Sera were also fractionated by Sephadex G200 chromatography to determine the proportions of IgM and IgG anti-BSA antibodies.

Complement determinations.—Total haemolytic complement (CH50) was measured by the kinetic turbimetric method of Lachmann and Hobart (1978). C3 values were monitored by single radial immunodiffusion.

IC clearance analysis.—The primary or Day 8 clearance of ^{125}I-BSA following immunization with BSA linked to red cells, was examined by following the disappearance of ^{125}I-BSA precipitable in 10% trichloroacetic acid (TCA) or 50% saturated ammonium sulphate (SAS). In addition, the proportion of ^{125}I label that remained protein-bound in each sample and indicating the extent of IC catabolism was measured by precipitation in 10% TCA. The proportion of ^{125}I-BSA that was complexed to antibody in each sample giving the concentration of IC was determined by precipitation in 50% SAS (Farr assay).

Gel filtration of IC formed in vivo.—The serum samples obtained at various intervals after injection of ^{125}I-BSA were analysed for IC by chromatography on Sepharose 4B.

RESULTS

Primary clearance of ^{125}I-BSA in normal rabbits

The clearance of TCA-precipitable ^{125}I-BSA in control rabbits is shown in Fig. 1. Immune clearance commenced on Days 5–6 and was complete by Day 9. The proportion of ^{125}I-BSA that was bound to antibody in each sample started to increase on Day 5 and reached a peak on

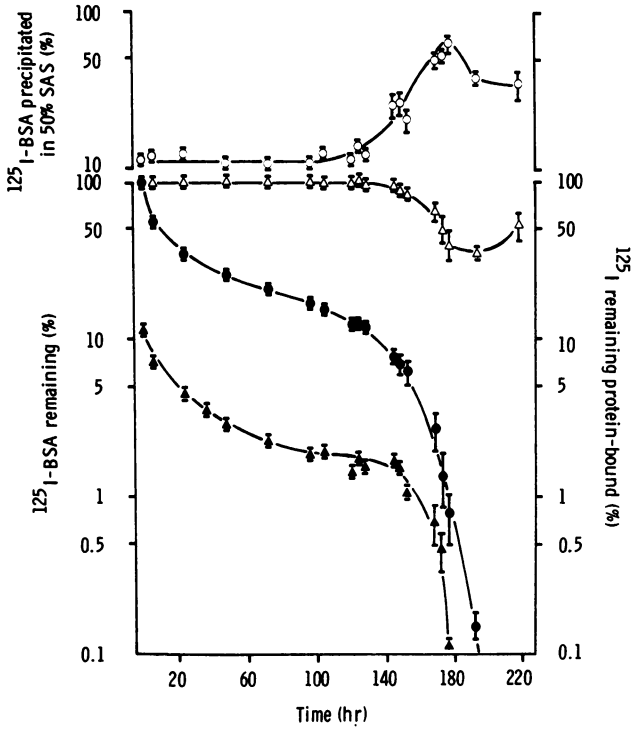

FIG. 1.—Semi-logarithmic plot of immune elimination of ^{125}I-BSA in normal rabbits immunized with BSA-coated erythrocytes. Clearance of ^{125}I-BSA precipitable in 10% TCA (●) or 50% SAS (▲). Proportion of ^{125}I precipitable in 10% TCA (△) or ^{125}I-BSA precipitable in 50% SAS (○). Values represent mean ± s.e. of 3 rabbits.

Day 7 in 2 rabbits (R93, R94) and Day 8 in the third (R92). The individual rabbits had antigen-binding values of 47, 61 and 100% with a mean value of 64%. The clearance of ^{125}I-BSA specifically bound to antibody began on Day 6 and finished on Days 8–9 (Fig. 1). The commencement of specific IC clearance was thus delayed about 12 h compared to the clearance of TCA-precipitable ^{125}I-BSA.

The amount of free ^{125}I appearing in the circulation, indicating catabolism of the cleared IC, was minimal (*i.e.* >95% protein-bound) over the first 5 days. Thereafter, the proportion of label remaining protein-bound decreased steadily as immune elimination proceeded. A substantial degree of variation was noted between individual rabbits in clearance rates and release of free ^{125}I. The rank order of rabbits with respect to the rate of elimination of the IC was R94, R93, R92. This order was reversed with respect to the proportion of ^{125}I remaining protein-bound, the lowest values found being 38, 31 and 15% for R92, R93 and R94 respectively. Towards the completion phase of elimination, the amount of free label decreased, and the protein-bound ^{125}I values returned to near pre-immune elimination levels. Thus, although variation between individual animals was noted, the rank order was consistent in that the animal forming most IC also cleared the IC most rapidly and released the greatest amounts of free ^{125}I.

Anti-BSA antibodies in normal rabbits

Antibodies to BSA started to appear in substantial amounts on Day 5, with the titres increasing for a further 3 or 4 days. The highest titre found before Day 5 was 1/256, while the highest found during the primary IgM response was 1/8096. After

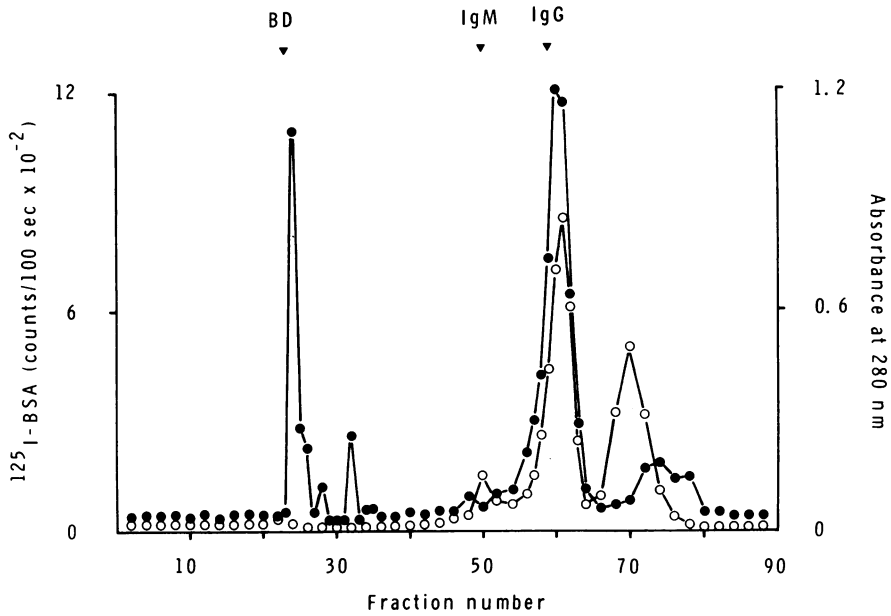

FIG. 2.—Sepharose 4B fractionation of rabbit serum obtained during immune elimination of ^{125}I-BSA in normal rabbits immunized with BSA-coated erythrocytes. ^{125}I-BSA (●); Absorbance at 280 nm (○). Mol. wt markers—Blue dextran (BD), IgM and IgG.

reduction and alkylation, the antibody activity of Days 5–9 serum samples was almost completely abolished, *e.g.* titres of 1/8096 were reduced to 1/8 or less after treatment. A steady rise in the amount of antibody resistant to DTT and IA treatment, presumed to be IgG, took place from Day 9 onwards. The titres observed, however, were still very low compared to peak values of total antibody on Day 7. Thus, the maximum IgG titre found during immune elimination was 1/32, while the overall maximum IgG titre was 1/256. The latter values were observed several days after immune elimination had been completed. Similarly, fractionation of sera on G200 columns showed that most of the antibody activity resided in the excluded macroglobulin peak.

Fractionation of IC on Sepharose 4B

The molecular size profile of IC formed during the immune clearance phase was obtained by fractionation of sera on Sepharose 4B. The results obtained from one rabbit serum are shown in Fig. 2. About 30% of ^{125}I-BSA was complexed in this 148 h post-injection sample, and the majority of these soluble IC were excluded from the 4B gel indicating that they were very large with a mol. wt of at least 20×10^6 daltons. The proportion of complexed ^{125}I-BSA to the total ^{125}I-BSA present in the sample thus indicated that the IC at this stage were in slight antigen excess.

Primary clearance of ^{125}I-BSA in CVF-treated rabbits

The results of C3 depletion by CVF on the clearance of ^{125}I-BSA is shown in Fig. 3. The TCA-serum clearance curves obtained were similar to those in the control animals, though the CVF-treated animals started clearing the antigen 1 day earlier on Day 5. Clearance of antigen precipitable in 50% SAS commenced 1 day later on Day 6, a lag similar to that observed in the control animals (Fig. 1).

The amount of free ^{125}I liberated from the IC following catabolism was slightly lower in the CVF-treated animals com-

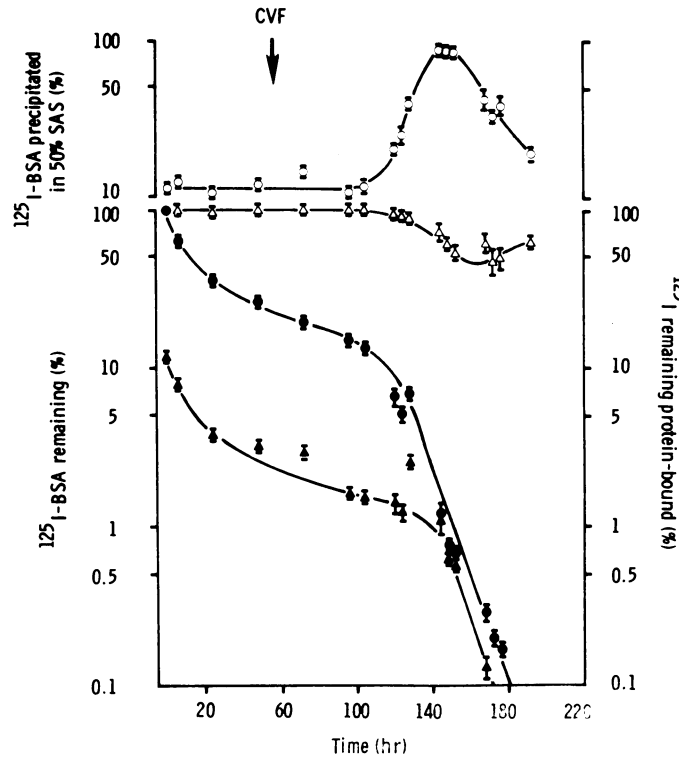

FIG. 3.—Semi-logarithmic plot of immune elimination of ^{125}I-BSA in CVF-treated rabbits immunized with BSA-coated erythrocytes. Clearance of ^{125}I-BSA precipitable in 10% TCA (●) or 50% SAS (▲). Proportion of ^{125}I precipitable in 10% TCA (△) or ^{125}I-BSA precipitable in 50% SAS (○). Values represent mean ± s.e. of 3 rabbits.

pared with the controls (Fig. 3). The lowest value obtained for the proportion of protein-bound ^{125}I was 35% on Day 7, while the mean value for the group was 46%. However, more of the antigen was complexed when analysed by the Farr assay, such that mean peak values of 85% were observed (Fig. 3). The peak also occurred earlier in the CVF-treated animals (Day 6) compared to control rabbits (Day 7).

The kinetics and magnitude of the IgM anti-BSA response were similar to the controls, with reduction and alkylation causing a drop in titre from 1/8096 to 1/32. However, only a small rise in IgG titre was found with maximum values remaining at 1/32.

Primary clearance of ^{125}I-BSA in ^{131}I-ovalbumin-treated rabbits

Injection of ^{131}I-ovalbumin 4 days after the initial injection of ^{125}I-BSA, resulted in marginal inhibition of BSA clearance rates (not shown). A slight delay was found following commencement of TCA-precipitable ^{125}I-BSA elimination which persisted until completion of clearance. For example, on Day 8 0·1 and 0·5% of the BSA injected remained in the blood in the control and ovalbumin-treated animals respectively.

The highest values found for IC formation by the Farr assay ranged between 61–90% for individual rabbits, with a mean of 67% for the group. These results were similar to those obtained with the

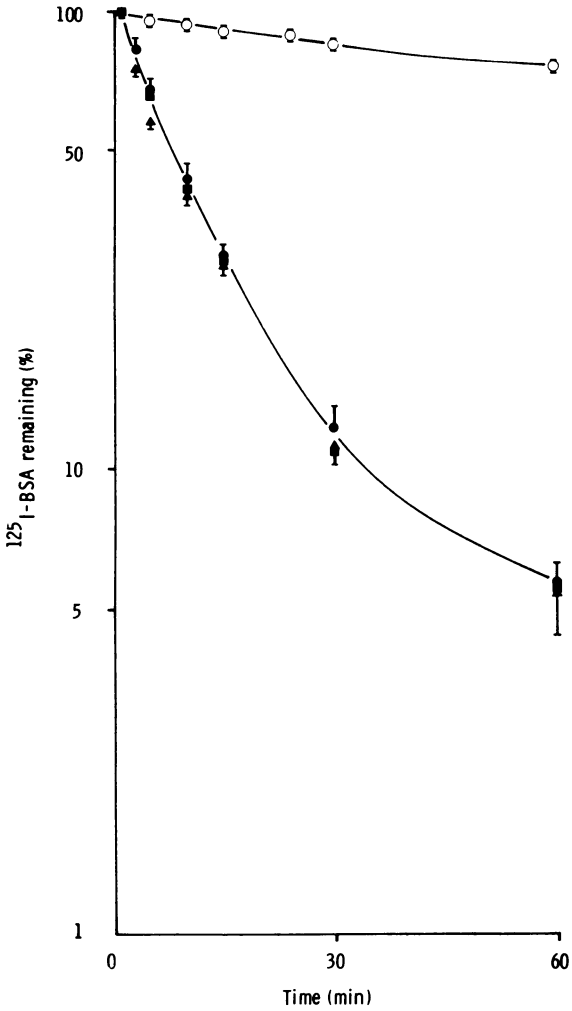

FIG. 4.—Semi-logarithmic plot of blood clearance of TCA-precipitable ^{125}I-BSA injected into rabbits 8 days after initial immunization with BSA-coated erythrocytes. Normal controls (●); CVF-treated (▲); ovalbumin-treated (■). Clearance of ^{125}I-BSA in unimmunized rabbits (○). Values represent mean ± s.e. of 3 animals in each group.

controls. The lowest values for protein-bound ^{125}I were slightly higher (45, 46 and 53%) than the controls, and these were obtained at about the same times (Day 8).

The *in vivo* concentration of ^{131}I-ovalbumin about 2 days after injection was approximately 1 mg/ml, and gradually declined from this value over the period of observation. The corresponding concentration of ^{125}I-BSA at this time was about 450 ng/ml of blood. There was therefore a 2000-fold excess of ^{131}I-ovalbumin over ^{125}I-BSA. Since no inhibition of ^{125}I-BSA clearance was noted, and no deviations occurred in the linear catabolic phase of ^{131}I-ovalbumin clearance, it would appear that the *in vivo*-generated IgM anti-BSA antibodies did not cross-react with the injected ovalbumin.

Day 8 clearance of ^{125}I-BSA in immunized rabbits

The clearance rates for ^{125}I-BSA injected into the blood of rabbits on Day 8 of the primary immune response to BSA-coated red cells is shown in Fig. 4. Rapid clearance of TCA-precipitable BSA was found in control, CVF-treated and ovalbumin-treated animals, compared to the clearance velocity of BSA in unimmunized rabbits. About 7% of the injected BSA remained in the circulation of immunized animals, whereas the values in unimmunized rabbits were in the region of 80%. The differences in clearance rates between the 3 experimental groups were not statistically significant. Similar results were found with the clearance of BSA specifically bound to antibody (not shown). After 1 h, about 3% of the injected BSA could be precipitated in 50% SAS from the blood of control animals, while mean values of about 4 and 5% were obtained from CVF-treated and ovalbumin-treated rabbits respectively.

Substantial but comparable amounts of free ^{125}I were released into the circulation by the 3 groups of animals during clearance of the IC, with values less than 20% protein-bound ^{125}I being observed (Fig. 5). The proportion of ^{125}I-BSA complexed in each serum sample was between 90–100% at time zero in all 3 groups, though this decreased to 60–80% over the period of observation.

DISCUSSION

In analysing the role of circulating IC in human disease, several studies have

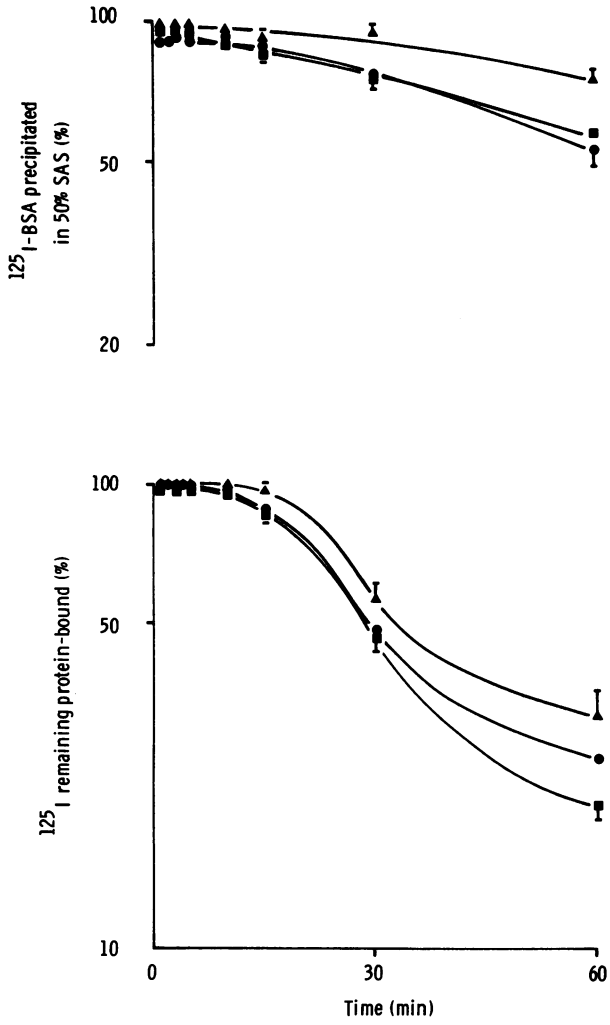

FIG. 5.—Semi-logarithmic plot of proportion of ^{125}I-BSA precipitable in 50% SAS (top panel), and proportion of ^{125}I precipitable in 10% TCA (bottom panel) in rabbits immunized with BSA-coated erythrocytes. Normal controls (●); CVF-treated (▲); ovalbumin-treated (■). Values represent mean ± s.e. of 3 animals in each group.

focused on the mechanisms involved in their recognition and clearance by the reticuloendothelial system (RES). Most reports have dealt with the interaction between soluble IgG.IC and RES receptors facilitating their elimination from the blood. The results of these studies have shown that IgG.IC are cleared primarily *via* Fc receptors located in the liver, and that the antigen/antibody ratio and extent of lattice formation are important determinants of their fate *in vivo* (Mannik *et al.*, 1971; Finbloom and Plotz, 1979).

In contrast, there have been few attempts to devise animal model systems for the study of soluble IgM.IC, and consequently little information is available on their normal physiological behaviour or their pathological potential. In previous reports, we described 2 different animal models for the characterization of the factors involved in the physiological clearance of soluble IgM.IC. In the first model, the clearance kinetics of soluble preformed IgM.IC in various antigen/antibody ratios were studied in complement replete and depleted rabbits (Harkiss and Brown, 1981). The results showed that IgM.IC close to equivalence were cleared with a greater velocity than those in antigen excess, and that CVF-treatment did not appreciably delay their removal from the blood.

In the second model, passive induction of *in vivo*-generated IgM.IC was analysed to determine the role of antigen/antibody ratio and the effect of C3-depletion on clearance velocity. In this model, rapid formation of IgM.IC was initiated by injection of IgM antibodies into rabbits with circulating antigen. This procedure resulted in sudden disturbance of the antigen plasma/tissue equilibrium to produce IgM.IC initially at equivalence, then progressively towards moderate and far antigen excess. The results obtained confirmed those found with preformed IgM.IC in that clearance was not inhibited by prior C3-depletion (Brown and Harkiss, 1981). However, this model represents the reverse of the usual course of events whereby antibody levels, initially low and forming antigen excess IC, gradually increase until a state of equivalence, then antibody excess is reached. Thus, while useful and yielding much valuable information, i.v. injection of pre-formed IgM.IC or of a bolus of IgM antibody represent highly artificial procedures, and are un-

likely to have a physiological or pathological counterpart *in vivo*.

In the present report, a more natural model was investigated. This involved following the elimination of an antigen during a primary IgM response in rabbits, or after introduction of antigen into pre-immunized animals. The main limitation of this approach at the outset was the possible contribution of any IgG antibodies present in the response to the antigen clearance. In an attempt to minimize this contribution, enhanced IgM responses were engineered by immunizing the animals with antigen in particulate form (Torrigiani and Roitt, 1965).

The results of analysing the antibody response showed that small amounts of IgG antibody were present during the immune elimination phase (Days 7–9). Fractionation of the *in vivo*-generated IC by gel chromatography showed they were both soluble and very large, with mol. wts greater than 20×10^6 daltons. This data is consistent with the molecular size of IgM.IC produced artificially *in vitro* using purified antibodies (Harkiss and Brown, 1981). Thus, while IgG antibodies were undoubtedly present during the immune elimination period, the results are consistent with the bulk of the antigen being cleared via IgM antibodies.

The results of the clearance studies performed here show that IC formed *in vivo* during immune elimination are cleared rapidly by mechanisms that are independent of C3b-fixation, and confirms conclusions reached with the other rabbit models (Harkiss and Brown, 1981; Brown and Harkiss, 1981) and rat IgM.IC clearance models (Day *et al.*, 1980). The data obtained from the primary clearance model also confirms the observation made on the *in vivo* handling of pre-formed IgM.IC that CVF-treatment slightly accelerates rather than inhibits clearance of IgM.IC, though this effect was not seen in the Day 8 model.

The reasons why CVF-treatment can induce accelerated clearance of IgM.IC are not clear, though it is possible that lack of complement results in an inability to "solubilize" pre-formed IgM.IC (Miller and Nussenzweig, 1975), or an inhibition of IgM.IC formation *in vivo* (Schifferli, Bartolotti and Peters 1980). The net effect of these processes would normally be to reduce the size of the IC present, and if prevented would allow larger IC to circulate and be presented to the RES. The accelerated uptake of large over small IC by the RES has been demonstrated by several investigators (Weigle, 1958; Mannik *et al.*, 1971; Plotz *et al.*, 1979). However, accelerated clearance *via* stimulation of the RES by C3b generated in serum cannot be excluded as an explanation for these observations.

The lack of effect of CVF-treatment on the clearance of IgM.IC observed by us here and previously, and with IgG.IC by others (Mannik *et al.*, 1971), raises the question of the efficacy of this procedure in completely depleting the animals of functional C3. It has been established that most complement components including C3 are synthesized by tissue macrophages in the liver (Colten, 1976). This local production of C3 and alternative pathway components may provide enough to opsonize small amounts of soluble IgM.IC in the blood and induce immediate uptake *via* C3b receptors.

However, CVF-treatment has been shown to reduce C3 levels enough to completely block uptake of IgM-coated red cells (Schreiber and Frank, 1972), though it may be argued that IgM-coated red cells require a high density of C3 molecules on their surfaces for efficient uptake by the RES, possibly due to the topography of membrane C3 receptors (Landen, Strunkheide and Dierich, 1978). Other studies have shown that increasing the density of IgM antibodies on red cells results in significant uptake and phagocytosis in the absence of complement (Mantovani, 1981). Phagocytosis of the IgM-coated red cells required the prior "activation" of the macrophages by glycogen, whereas uptake *via* the IgM Fc receptor did not depend on this process. In the present study, the

amounts of IC generated *in vivo* were sufficiently large to reduce CH50 values to 10% or less in the control animals, and therefore cannot be considered as being "trace amounts". These observations tend to support the contention that soluble IgM.IC in rabbits are cleared *via* C3b-independent mechanisms, possibly involving "activation" of RES IgM Fc receptors.

Recently, other investigators have presented evidence which showed that soluble IgM.IC in rats were cleared *via* mannosyl receptors in the RES (Day *et al.*, 1980). Their studies indicated that the rat antibodies possess obligosaccharides which are only exposed after antigen-binding, and that the *in vivo* clearance of soluble IgM.IC could be inhibited by treatment with yeast mannan or ovalbumin, molecules possessing similar mannosyl groupings to the complexed IgM antibodies.

To determine if such a signalling system was operating in the rabbit, we injected a large amount of ovalbumin into the circulation just before the immune elimination of BSA. The results obtained with both model variants showed that little or no inhibition of IgM.IC clearance occurred in the ovalbumin-treated animals, and indicate that in rabbits mannosyl groups on IgM antibodies play little if any part in the clearance of IgM.IC. Recent reports demonstrating clearance of IgG.IC *via* sugar residues present on the antigen (Rifai *et al.*, 1982) suggest however that IgM.IC with the appropriate antigens may be cleared *via* sugar receptors in the RES.

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