THE ENHANCEMENT OF 19S ANTIBODY PRODUCTION BY PARTICULATE ANTIGEN*

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In mammalian species, the injection of antigen in adequate amounts gives rise initially to the production of high molecular weight antibody of the 19S class, to be superseded in the majority of instances by the synthesis of 7S antibody (Bauer and Stavitsky, 1961; Uhr, 1964). The continued presence in many arthritic patients of high titre 19S antibodies thought to be directed against the subject's own slightly altered γ-globulin, led us to consider possible factors which might enhance the macroglobulin phase of antibody synthesis.

The ability of heterologous red cells to evoke a somewhat sustained 19S response (Stelos, Taliaferro, and D'Alesandro, 1961), the production of rheumatoid-like factors in rabbits by hyperimmunization with bacteria (Eyquem *et al.*, 1958; Milgrom and Witebsky, 1960; Abruzzo and Christian, 1961), and the preferential reaction of human rheumatoid factors with γ-globulin bound either in the form of an immune complex or coated on particles such as latex, suggested that a comparison of macroglobulin antibody production occasioned by the injection of a given antigen in different physical forms might give some insight into this problem. In particular we have compared the responses to antigens given in aqueous solution and in a particulate form bound to an inert carrier.

The results of preliminary experiments were published previously (Roitt, Torrigiani, and Doniach, 1963).

Materials and Methods

Animals.—Adult rabbits of both sexes weighing between 2.5 and 4 kg were used.

Sera.—Blood was taken from the ear vein. Serum was usually fractionated immediately or stored at −20°C.

Antigens.—Human thyroglobulin was prepared by salting out with ammonium sulphate (Derrien, Michel, and Roche, 1955). A solution of 20 mg/ml in phosphate-buffered saline was spun at 4000 RPM for 10 minutes before injection. Human γ-globulin prepared by ether

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Immunization.—

(*a*) *Aqueous protein solution:* Three animals were given single intravenous injections of 100 mg thyroglobulin, one was given 40 mg and two others 30 mg. The schedule for multiple injections was as follows: 15 mg of thyroglobulin or γ-globulin on day 1 and a further 15 mg every 5 days.

(*b*) *Antigen-antibody complex:* A precipitating Hashimoto serum was mixed in equivalent proportions with 60 mg of thyroglobulin. After incubation at 37°C for 30 minutes and 4°C overnight, the immune precipitate was spun down, washed twice with cold saline and injected intravenously into 2 rabbits as a saline suspension.

(*c*) *Alum precipitate:* 80 mg of thyroglobulin dissolved in 5.0 ml of saline was mixed with 3.0 ml of 1 M sodium bicarbonate and 5.0 ml of 10 per cent potassium alum slowly added with stirring. The precipitate was spun down, washed with M/15 phosphate buffer pH 7.2, and injected intravenously into 2 rabbits as a suspension in that buffer.

(*d*) *Freund's adjuvant:* A solution of 320 mg of thyroglobulin in 8 ml saline was emulsified with 24 ml of complete Freund's adjuvant consisting of 5 parts bayol F, 4 parts falba, and 1 part arlacel A, and containing 2 mg/ml heat-killed human tubercle bacilli. The emulsion was injected into 8 rabbits using intradermal, subcutaneous, and intramuscular routes.

(*e*) *Polyacrylic resin particles:* To each ml of an 11 per cent suspension of 0.8 μ diameter particles (Bofors, Nobelkrut, Sweden) was added 50 mg of either thyroglobulin or human γ-globulin in 2 ml 0.02 M phosphate buffer pH 6.3. The suspension was incubated at 50°C for 1 hour and left at room temperature overnight. The particles were spun at 4000 RPM for 15 minutes, washed twice with saline and resuspended in the original volume of saline. Each ml of final suspension contained 9 to 12 mg of thyroglobulin or 7 mg of γ-globulin. Each rabbit was injected with 3 ml of suspension intravenously and boosted where indicated with 2 ml volumes every 5 days for 3 weeks.

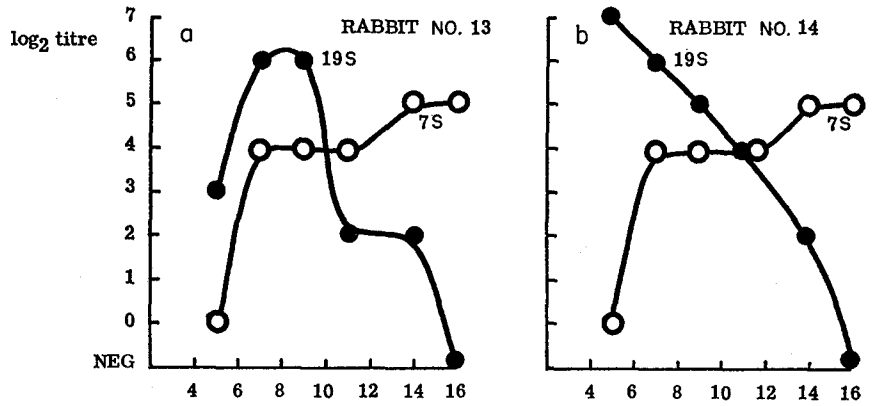
(*f*) *Coated red blood cells:* The animal's own erythrocytes were used and treated with pyruvic aldehyde as described by Ling (1961). To each ml of a 50 per cent suspension of treated cells was added 50 mg of either thyroglobulin or γ-globulin dissolved in 1 ml of 0.02 M phosphate buffer pH 6.3. The mixture was incubated for 2 hours at 50°C, the cells spun down and washed twice with saline and finally made up to a 10 per cent suspension in saline, each ml containing roughly 0.2 mg of antigen. Each rabbit received 2 ml of this cell suspension intravenously. Volumes greater than 3 ml often led to the death of the animal.

Serological Tests.—Thyroglobulin antibodies were detected by the tanned cell haemagglutination technique employing preserved thyroglobulin-coated erythrocytes (Fulthorpe *et al.*, 1961). Antibodies to γ-globulin were detected in a similar way using red cells preserved with pyruvic aldehyde and coated with 1 mg/ml γ-globulin as described above for thyroglobulin. The tests were carried out in perspex microagglutination trays using the spiral loop method of Takatsy for serial dilutions as modified by Sever (1962).

Treatment with 2-Mercaptoethanol.—The sample to be tested was left for 48 hours with an equal volume of 0.2 M 2-mercaptoethanol in 0.05 M phosphate pH 7.2 containing 0.1 M sodium chloride. The sulphydryl compound was removed by dialysis against repeated changes of saline for 24 hours.

Column Chromatography on DEAE-Cellulose.—Both the DEAE-cellulose (Serva-Entwickslung, Heidelberg) and the serum to be fractionated were equilibrated with 0.02 M phosphate pH 6.3. The small amount of precipitate which formed in the serum was spun off. Stepwise elution with phosphate buffers of increasing molarity was employed finishing with 1 M sodium chloride. Elution with each buffer was continued until no protein was detectable. The fractions were concentrated in dialysis tubing against negative pressure and analysed ultracentrifugally on the same day.

Ultracentrifugation.—A sucrose gradient consisting of 5 0.9 ml fractions of 10, 14, 18, 22, and 26 per cent sucrose in 0.05 M phosphate buffer pH 7.2 was used. 0.15 ml of serum diluted to 0.5 ml with saline was layered over the gradient and spun in the SW39 head of the Spinco model L at 35,000 RPM for 15 hours with a fluorescein-labelled 7S γ-globulin marker. Where the relative proportions of 7S and 19S antibodies in a given serum were to be determined, the first 1.8 ml from the bottom of the tube was taken for the 19S fraction, the next 0.5 ml was discarded and the subsequent 1.8 ml analysed as the 7S fraction (*cf.* Torrigiani and Roitt, 1963). When more detailed analysis was required, approximately 30 fractions were collected from the tube at the end of the run and analysed individually. The reliability of the fluorescent marker as a guide to the location of 7S antibodies was confirmed by the observation that the fluorescence coincided with the peak of 7S incomplete anti-D antibodies. Further, when centrifugation was carried out under these conditions, the marker was well separated from the natural rabbit anti-sheep cell haemolysins which have a sedimentation constant of 19S.



FIGS. 1 *a* and 1 *b*. 7S and 19S antibody titres in 2 individual rabbits following a single intravenous injection of thyroglobulin solution.

RESULTS

Response of Rabbits to a Single Intravenous Injection of Aqueous Antigen.—The titres of the 19S and 7S antibody fractions in the sera of 2 individual rabbits given 100 mg of human thyroglobulin are shown in Figs. 1 *a* and 1 *b*. Five days after the injection, 19S antibodies predominated but by the 16th day these were no longer detectable and were replaced by 7S antibodies. This decline in macroglobulin antibody synthesis was a consistent finding in a further four animals. The same sequence of immunological events was observed in two rabbits injected with an antigen solution which had been spun previously at 20,000 RPM (Spinco L; "40" head) for 20 minutes to remove possible aggregates.

Comparable results were obtained after a single intravenous injection of a human γ-globulin solution. By the 18th day, no haemagglutinating antibodies could be demonstrated in the macroglobulin fraction in 5 rabbits studied whereas 7S titres of 32 to 256 were observed.

Response to Single Injections of Antigen in Differing Physical States.—Sera from rabbits injected with comparable amounts of human thyroglobulin adsorbed to an alum precipitate, in the form of a complex with human autoanti-

TABLE I

Macroglobulin Antibody Response to Single Injection of Thyroglobulin in Different Physical Forms at 15 Days

Rabbit No.	Physical state of antigen	Macroglobulin fraction	
		Titre*	Titre as per cent of total antibody
R8	Aq solution	<5	<10
R9	" "	<5	<5
R28	" "	1	1
R13	" "	2	6
R14	" "	2	6
RE	" "	2	6
RF	" "	2	6
R19	" "	32	10
F3	Alum precipitate	16	1.5
F4	" "	32	1.5
F1	Antigen-antibody complex	16	5
F2	" " "	16	5
R36	Freund adjuvant	16	1
F11	" "	32	3
F12	" "	32	1.5
F14	" "	32	3
F15	" "	32	1.5
F13	" "	64	3
F16	" "	64	3
R37	" "	64	3
R39	Bound to acryl particles	2	11
RC	" " " "	2	11
RD	" " " "	2	11
F6	" " " "	4	80
R24	" " " "	4	70
R29	" " " "	8	6

* Titres are not corrected for a dilution factor of 12 relative to the original serum.

body, emulsified in Freund's complete adjuvant or coated on to the surface of acrylic resin particles, were fractionated by zone ultracentrifugation. Macroglobulin antibody titres tended to be raised in animals treated with thyroglobulin on an alum precipitate, as an immune complex or in Freund adjuvant but

were no higher when expressed as a fraction of the total serum titre relative to animals injected with aqueous antigen (Table I). On the other hand, antigen coated on acryl particles increased the contribution of 19S antibodies to the total agglutinating titre of the serum. However, since the antibody titres were low, the effect of repeated injections was investigated.

Effect of Boosting Injections of Antigen in Particulate and Aqueous Forms.— The antibody response to single and repeated injections of thyroglobulin on acryl particles is shown in Table II. The proportion of the serum titre contributed by the macroglobulin fraction was similar in the two groups, but animals receiving multiple injections had much higher antibody levels. Boosting with particulate antigen increased the titre of 19S antibodies 10- to 20-fold when compared with levels reached in animals injected repeatedly with the same amount of antigen in soluble form (Fig. 2 *a*); in contrast, the 7S antibody titres show no significant difference (Fig. 2 *b*). Expressed as a percentage of the total

TABLE II

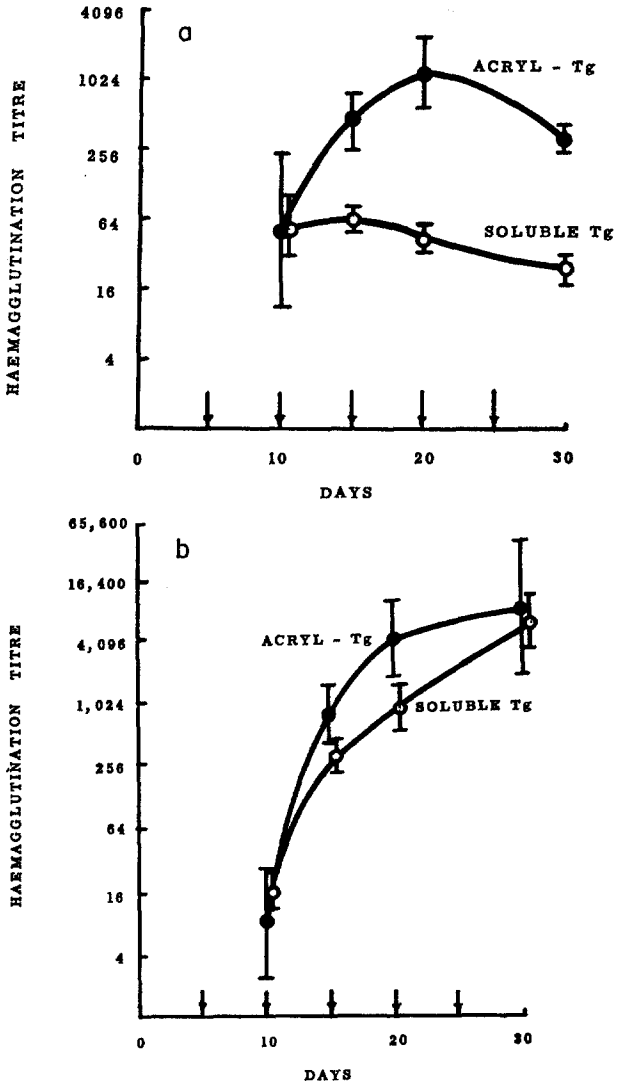
Comparison of Macroglobulin Antibody Response at 15 Days to Single and Repeated Injections of Thyroglobulin Coated on Acryl Particles

Injection	No. animals	Macroglobulin fraction	
		Log_2 titre*	Titre as per cent of total
Single	6	1.7 ± 0.33	31.5 ± 13.9
Boosted every 5 days	5	9.4 ± 0.75	35.2 ± 9.1
P	—	<0.01	N.S.D.

* Mean ± standard error of the mean.

serum titre, the contribution of the macroglobulin antibodies fell with time but the mean values were significantly higher than in the control group given aqueous antigen and this difference was maintained even after 2 months (Table III). This stimulating effect was not observed when uncoated resin particles and a solution of thyroglobulin were administered at different times; indeed the mean 19S log_2 titre of 3.7 at 21 days suggested a possible inhibition by blockade of the reticuloendothelial system. The haemagglutinating activity of antibodies produced in response to immunization with acryl-bound thyroglobulin could be completely inhibited by absorption with an aqueous solution of the antigen.

These experiments were repeated with human γ-globulin as antigen with entirely comparable results; titres of 19S antibody were on average four log_2 units higher in the rabbits given acryl-bound antigen as compared with the group receiving soluble antigen (Table IV). Furthermore, at 21 days the titre of 19S antibodies was actually higher than that of the 7S fraction, in contrast with the results obtained with acryl-thyroglobulin.



FIGS. 2 *a* and 2 *b*. Comparison of antibody responses in groups of rabbits receiving repeated injections of thyroglobulin either as a solution or coated on acrylic particles. The geometric mean titres for each group are plotted. The range of values obtained by calculating the standard error of the mean of the $\log_2$ titres for each group is given. Fig. 2 *a* Macroglobulin antibody response; Fig. 2 *b* 7S antibody response.

Four rabbits were injected with thyroglobulin coated on pyruvic aldehyde-treated cells with subsequent injections on days 5 and 10; the macroglobulin fractions of the sera taken at 15 days had $\log_2$ titres of 7, 8, 8, and 10 respectively which compare with the mean $\log_2$ titre of 5.5 ± 0.5 for the 7 animals boosted

with aqueous antigen. The titre of the macroglobulin fraction was equal to that of the 7S in 1 animal at this time and greater in the three other rabbits whereas it represented only 20 ± 7 per cent for the control group injected with aqueous antigen. Thereafter the 19S titre fell sharply and by 21 days, the mean titre in

TABLE III

Comparison of Macroglobulin Antibody Titre, Expressed as a Percentage of the Total Serum Titre, in Rabbits Given Boosting Injections of Thyroglobulin in Solution or Bound to Acryl Particles

Thyroglobulin injected	No. of animals	Macroglobulin antibody titre*				
		10 days	15 days	20 days	30 days	55 days‡
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
On acryl particles.....	7	85 ± 5	39 ± 13	18 ± 4	4 ± 1	4 ± 1
As aq. solution.....	7	75 ± 6	20 ± 7	5 ± 1	0.6 ± 0.2	0.9 ± 0.3
P.....		N.S.D.	N.S.D.	0.01	0.05	0.05

Boosting injections were given on days 5, 10, 15, 20, 27, and 50.

* Mean ± standard error of the mean.

‡ Only 3 animals in each group were studied at 55 days.

TABLE IV

Antibody Production in Response to Repeated Intravenous Injections of Human γ-Globulin in Solution or Bound to Acryl Particles

γ-Globulin injected:	No. of animals	Log_2 titre* of antibodies to γ-globulin			
		16 Days		21 Days	
		19S	7S	19S	7S
		16 days		*21 days*	
On acryl particles.....	6	5.5 ± 0.42	3.7 ± 0.45	8.0 ± 0.46	6.5 ± 0.61
As aq. solution.....	6	3.0 ± 0.26	3.7 ± 0.36	4.1 ± 0.40	6.3 ± 0.54
P.....		<0.01	N.S.D.	<0.01	N.S.D.

Boosting injections were given on days 5, 10, and 16.

* Mean ± standard error of the mean.

a group of 9 animals was no higher than in the rabbits boosted with soluble thyroglobulin.

Biochemical Characteristics of Antibodies Produced by Antigen Coated on Acryl Particles.—The serum from a rabbit given boosting injections of acryl-bound thyroglobulin, taken 21 days after the primary injection, was chromatographed on a DEAE-cellulose column and four fractions eluted stepwise with the buffers indicated in Table V. Fraction I contained high titre 7S γ_2-globulins stable to 2-mercaptoethanol. Similar antibodies of lower titre but with γ_1-mobility were

found in fraction II. The two final fractions contained a complex mixture of γ_1-antibodies of 19S, intermediate and 7S class, the latter being present in low titre; the distinct sedimentation properties of the 19S and intermediate size antibodies was demonstrated by recentrifuging the fractions shown in Fig. 3. Reduction of fractions III and IV with mercaptoethanol led to substantial loss of haemagglutinating activity. The antibodies were not destroyed by heating to 63°C for 20 minutes although natural sheep cell haemolysins are inactivated under these conditions.

Similar studies were carried out using the serum from a rabbit receiving mul-

TABLE V

DEAE-Cellulose Chromatography of Sera from Rabbits Given Boosting Injections of Thyroglobulin as a Solution or Bound to Acryl Particles

Fraction No.	Eluted with	Radioimmuno-electrophoretic mobility of antibodies	Sedimentation analysis	Anti-body* titre	Titre* after:	
					2-mer-capto-ethanol	Heating to 63°C for 30 min
Serum from rabbit injected with acryl-bound thyroglobulin						
I	0.02 M PO_4 pH 6.3	γ_2	7S	2048	2048	2048
II	0.1 " "	γ_1	7S	128	128	128
III	0.15 " "	γ_1	19S, Int., 7S	128	8	128
IV	1 N NaCl	γ_1	19S, Int., 7S	1024	16	1024
Serum from rabbit injected with aqueous thyroglobulin						
I	0.02 M PO_4 pH 6.3	γ_2	7S	1024	1024	1024
II	0.1 " "	γ_1	7S	16	16	16
III	0.15 " "	γ_1	19S, (Int.)	2	0	0
IV	1 N NaCl	γ_1	19S, (Int.)	16	0	16

* Fractions concentrated to volume of original serum sample.

tiple injections of aqueous thyroglobulin solution (Table V). The major part of the antibody activity was recovered in the breakthrough peak of 7S γ_2-globulins. Relatively low titres were found in the 7S γ_1-globulins, the last two fractions containing 19S proteins, and questionable amounts of antibody with intermediate sedimentation characteristics (Fig. 3).

DISCUSSION

Rabbits respond initially to the intravenous injection of human thyroglobulin solution by the production of macroglobulin antibodies but this is superseded within 2 to 3 weeks by the synthesis of 7S immunoglobulins, a sequence of events which has previously been described for the majority of antigens studied. Furthermore the production of high molecular weight antibody was not depend-

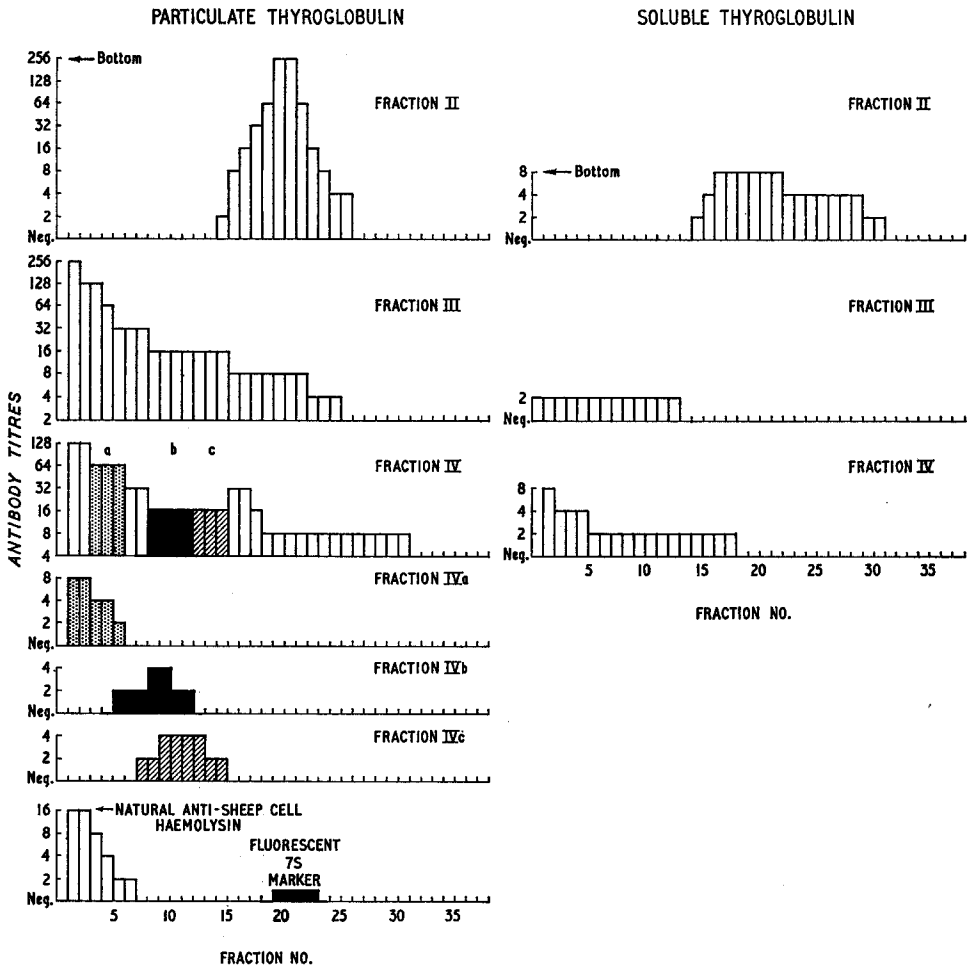


FIG. 3. Ultracentrifugal analysis of DEAE-cellulose chromatography fractions of sera from rabbits given boosting injections of thyroglobulin as a solution or bound to acryl particles (*cf.* Table V). Each histogram represents the distribution of antibody activity within the density gradient after centrifugation of the appropriate fraction under standard conditions. The base of the tube is represented on the left in each case. Subfractions obtained after centrifuging fraction IV from the serum of the animal receiving particulate antigen were grouped as indicated by the shading and respun to give the profiles shown under fractions IV*a*, IV*b*, and IV*c*. The distribution of 19S and 7S markers spun under identical conditions are shown.

ent upon the presence of protein aggregates in the immunizing inoculum since the same results were obtained by injection of protein solution which had previously been spun at 400,000 *g* minutes.

The level of 19S thyroglobulin antibody could be maintained by boosting

with soluble material which presumably leads to the continual recruitment of new antibody-forming cell lines. However, coating the antigen on acrylic resin particles led to a 10- to 20-fold increase in macroglobulin antibody agglutinating titre although 7S antibody levels were unchanged. Thus immunization with the antigen in a particulate rather than a soluble form appears to enhance the production of 19S antibody without having any effect on 7S antibody synthesis. This phenomenon was reproduced when human γ-globulin was used as the antigen; titres of macroglobulin antibody were approximately 20-fold higher in rabbits given particulate as compared with aqueous antigen while the 7S titres remained comparable in the two groups. Also, the major part of the serum haemagglutinating activity in animals receiving γ-globulin–coated particles was in the heavy antibody fraction while 7S antibody predominated in the acryl-thyroglobulin recipients, suggesting that the chemical nature of the antigen may also influence the response. However, these results may not imply that the relative antibody concentration of the 19S fraction was greater than that of the 7S in the former group since the agglutinating power of macroglobulin antibodies against antigen-coated erythrocytes is known to be several times greater on a molecular basis. It was of interest that "intermediate" sedimenting and 7S γ_1-antibodies were also enhanced in the animals given particulate antigen.

Stimulation of macroglobulin antibody synthesis was also noted by Singer and his colleagues (1963) when they injected a variety of mammalian species with latex-bound γ-globulin. Further support for the view that an antigen in particulate form favours the production of high molecular weight antibody derives from comparison of the immunological response in the rat to injections of monomeric and polymeric *Salmonella* flagellin preparations; whereas the polymer induced the sequential formation of 19S and 7S antibodies, only the latter were detectable in animals injected with the monomer (Nossal *et al.*, 1964 *a*).

The events underlying the enhancement of macroglobulin antibody production are not fully understood. It is unlikely that they represent an effect of inert particles *per se* on the lymphoreticular system since the stimulation of 19S antibody was not seen in the present experiments when resin was injected independently of the antigen. The phenomenon could depend upon a different organ localization of the particulate antigen although there is no clear evidence of preferential synthesis of a particular immunoglobulin type in any one tissue. The transient macroglobulin antibody response to a single injection of most antigens is maintained only by further administration of antigen (Bauer, Mathies, and Stavitsky, 1963; Uhr and Finkelstein, 1963; Svehag and Mandel, 1964 *b*). The ability of acryl-bound antigen to enhance 19S production may be due to prolonged persistence of the antigen in macrophages as a result of its association with metabolically inert particles or perhaps to an increased uptake into these cells.

The relationship of 19S to 7S globulin-producing immunocytes is still uncertain and we do not know whether cells making 19S antibody switch over to producing 7S, or whether they exist as independent cell lines (Schoenberg *et al.*, 1965). That cells can switch over to the synthesis of 7S is suggested by the finding of both immunoglobulin types in a minor proportion of cells as determined by the double labelling fluorescent antibody technique (Mellors and Korngold, 1963) and by studies on single cells in microdroplets (Nossal *et al.*, 1964 *b*). If this hypothesis is correct, the increase in 19S levels without any change in 7S titres obtained in the present studies would not be explicable in terms of a stimulation of mitosis in 19S cells before their transformation to 7S production, since this would lead to a proportionate increase in 7S cells. Neither could an explanation be sought in terms of a prolongation of the life of 19S cells since this would lead to a lag in the appearance of 7S antibody which was not observed (Fig. 2 *b*).

Evidence can also be cited in favour of the other view, that populations of 19S and 7S cells may evolve independently. Although human spleen and lymph nodes showed occasional germinal centres staining for both immunoglobulins, Mellors and Korngold (1963) found that the majority of these centres were committed to the synthesis of one immunoglobulin class, either IgG, IgA, or IgM. Furthermore a number of different experimental circumstances have been described in which 19S and 7S antibody production can be influenced independently. For example, small doses of bacteriophage in guinea pigs (Uhr, 1964) or of poliovirus in rabbits (Svehag and Mandel, 1964 *a*) evoke the formation of 19S antibody alone while higher doses lead to subsequent 7S antibody synthesis. 7S not associated with prior 19S antibody formation has been found after injection of a monomeric *Salmonella* flagellin (Nossal *et al.*, 1964 *a*); conversely the *Salmonella* "O" antigen gives rise predominantly to 19S antibody (Bauer and Stavitsky, 1961). The differential effects of endotoxin and of x-irradiation on the kinetics of 19S and 7S production provide further evidence (Uhr and Finkelstein, 1963). Our studies fit most readily into a hypothesis allowing preferential stimlation of 19S cells independently of the 7S population.

A third possibility is that a proportion of 19S cells switch over to become 7S clones which sustain antibody production and provide "memory" cells, while the remainder continue as a 19S cell population, perhaps dividing but ultimately dying (Nossal *et al.*, 1964 *b*). On this basis, the present observations would require the further postulate that particulate antigens specifically stimulate the latter cells. A choice between these different possibilities cannot be made at the present time. It is hoped that further information on the interrelation between the different cell populations may be obtained by fluorescent antibody studies using antisera to specific immunoglobulins. In any event, it is realised that the complexity of the immunoglobulin system in the rabbit has been oversimplified in this discussion and consideration must ultimately be given at least to the two

types of 19S described by Svehag (1964), to intermediate sedimenting antibody (Rockey and Kunkel, 1962; Roitt *et al.*, 1963; Svehag, 1964; Onoue *et al.*, 1964), and to multiple forms of 7S γ-globulin.

SUMMARY

Injection of human thyroglobulin solution into rabbits gave rise to a transient 19S antibody response which could however be maintained by repeated administration of antigen. When the antigen was coated onto acrylic resin particles, the titre of 19S antibodies was increased nearly 20-fold whereas 7S antibody levels were unchanged. This selective enhancement of 19S antibody synthesis by particulate antigen was also seen using human γ-globulin. "Intermediate" sedimenting and 7S γ_1-antibodies were also increased in animals given particulate antigen. These phenomena may be due to prolonged persistence of the antigen in appropriate macrophages or perhaps to an increased uptake into these cells. The results are discussed in terms of the relationship between 19S and 7S globulin-producing cells.

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