THE EFFECT OF CROSS-REACTING ANTIGENS ON THE TOLERANT STATE

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Tolerance affects the specificity of the antibody response. This can be demonstrated by injecting an animal at birth with a macromolecule and thereafter studying the immune response to a different, but structurally related, macromolecule (1–8). Such treatment gives rise to antibodies, directed mainly against structures on the immunizing macromolecule which are not present on the tolerance-inducing substance. However, there is also reactivity with the tolerance-inducing macromolecule. This can be attributed to antibody molecules which are directed against configurationally altered protein determinants and which cross-react with the corresponding native determinants (8). Thus it appears that the tolerant state can be circumvented by injecting a cross-reacting antigen. On the other hand, the tolerant state might be prolonged by some of the determinants of a cross-reacting antigen. It is well known that the period of tolerance can be extended by the administration of the tolerance-inducing antigen during adult life (9). This must imply that tolerance to all or most of the individual determinants is thus prolonged. However, many such determinants are also present on a cross-reacting antigen and it would therefore seem reasonable to assume that tolerance to these shared determinants must be maintained by the injection of cross-reacting antigen. Thus two divergent results may develop from such immunization of a tolerant animal; (*a*) the formation of antibody which may terminate tolerance, and (*b*) the prolongation of tolerance by determinants which are common to the immunizing and tolerance-inducing macromolecules. This is not likely to play any part in *natural* tolerance, but may affect the results of model experiments, and hence needs to be analyzed if results of such experiments are to be related to the consequences of natural tolerance. The following experiments were designed to distinguish and if possible to disentangle the effect of these opposing tendencies.

Materials and Methods

Reagents

Human Albumin (HA).—Human albumin, Behringwerke "reinst" (Behringewerke, Marburg-Lahn, West Germany) was used throughout.

2-Mercaptoethanol was obtained from Eastman Organic Chemicals, Rochester, N.Y.

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Sodium radioiodide HA.^{131}I, cysteine-free was obtained from Charles E. Frosst, Montreal, Quebec, Canada.

Tannic acid was obtained from British Drug Houses, Toronto, Canada.

^{35}S-labeled sulfanilic acid was obtained from the Radiochemical Centre, Amersham, England.

Oxazolone.—2-phenyl 4-ethoxy methylene oxazolone was prepared by Dr. J. A. Stock of the Chester Beatty Institute, London, with and without ^{14}C label.

Immunological and Analytical Methods

Preparation of Modified Proteins, Azo Human Albumin (HA-D), and Oxazolonated Human Albumin [HA (Ox)].—Diazotized sulfanilic acid coupled to HA was prepared as previously described (1, 2). Three products were prepared which differed in the degree of coupling: HA-D_8, HA-D_{16}, and HA-D_{35} (8). HA covalently linked to oxazolone was prepared ($HA(Ox)_8$, $HA(Ox)_{38}$) as described by Yoshimura and Cinader (7). The properties of these compounds have been described (7, 8).

Iodinated Human Albumin (HA.^{131}I).—Iodination was carried out by a modification (1) of the method of Berson, Yalow, Schreiber, and Post (10).

Induction of Tolerance in Newborn Rabbits.—Newborn rabbits were injected intraperitoneally with HA dissolved in 0.15 M NaCl: the first injection was given within 12 hr of birth. A total of 20 mg of HA given in four injections was administered within 84 hr after birth.

Injection of Adult Rabbits.—Injection of protein dissolved in 0.15 M NaCl was made into the marginal vein of the ear. A course of injections consisted of three injections administered at intervals of 48 hr. Courses of injections were carried out during the following periods: 44–48, 69–73, 94–98, 119–123, 144–148, 169–173, 194–198, 219–223, and 244–248 days after birth. Animals were bled 5 and 11 days after the last injection of each course.

Agglutination.—Antibody levels were measured by Boyden's method (11) involving the agglutination of tanned sensitized sheep erythrocytes as described by St. Rose and Cinader (8).

Examination of the Specificity of Antibodies by Agglutination-Inhibition.—This method has been applied in earlier studies of acquired immunological tolerance (1, 7, 8).

Determination of the Agglutinating Capacity of Antisera after Preincubation with 2-Mercaptoethanol.—The procedure was based on that adopted by Uhr and Finkelstein (12). Serum was diluted 1:10 with 0.15 M NaCl and incubated in a final concentration of 0.1 M 2-mercaptoethanol at 37°C for 30 min. The agglutination titer was then determined in the presence of mercaptoethanol. As a control, the titer of untreated sera was measured in the same experiments.

Elimination Tests.—Animals to be injected with HA.^{131}I were given KI in their drinking water during the week preceding the injection. HA.^{131}I was injected into the marginal vein of one ear and blood was taken from the opposite ear at intervals of 24 hr. An equal volume of 20% trichloroacetic acid was added to the sera and the resulting precipitates were separated by centrifugation for 30 min at 2500 rpm. The radioactivity of the intact sera and of the precipitates obtained by treatment with trichloroacetic acid was determined. The log radioactivity, corrected for the half-life of ^{131}I, was plotted as a function of time.

The duration of the tolerant state was defined operationally in terms of the elimination test. Animals which eliminated HA.^{131}I in two phases were considered tolerant, and animals which eliminated the protein in three phases were considered to be nontolerant. All animals received the same relative quantity of HA.^{131}I (5.24 mg protein per kg body weight). From the elimination curve of animals which eliminate HA.^{131}I in two phases, we determined m_0 the apparent equilibrium concentration at zero time of antigen in the circulation. The value of m_0 was found by extrapolating the curve of the metabolic phase to zero time. This was found

to be fairly constant in different animals and showed a variation of less than 20%. We defined $t_{0.1}$ as the time in hours at which the circulating antigen is 0.1% of the amount of antigen m_0. The value of $t_{0.1}$ depends upon the onset of antibody formation.

RESULTS

Effect of Cross-Reacting Antigens on Tolerance Maintenance.—We have previously examined the duration of the tolerant state in rabbits injected with 20 mg of human albumin (HA) at birth. Maintenance of tolerance was defined by the diphasic elimination of iodinated human albumin (HA. ^{131}I). In 94% of animals tolerance was maintained for 84 days, in 51% of animals for 139 days, and by the 260th day it was lost in all animals (8). Immunization with chemically modified HA during the first 73 days of life did not cause any change in the duration of tolerance as judged by the elimination of HA. ^{131}I, injected on day 85 (7, 8). In the present study, we have examined the effect of cross-reacting antigens on the maintenance of tolerance. HA.^{131}I was injected into 135-day-old animals which received *p*-azosulfonic acid derivatives of HA(HA-D) between the 44th and 123rd day. We found that 95% (20/21) of the animals were tolerant (groups I-a, I-b, and I-c, Table I). This proportion of tolerant animals was greater than that found among animals injected at birth with HA but not injected with cross-reacting antigen ($P < 0.001$). This was also the case if administration of cross-reacting antigen was confined to two courses of injections given between the 44th and 73rd day ($P = 0.003$), but was not found when the two courses were given between the 94th and 123rd day ($P > 0.05$). Injections during this later period, either with HA or HA-D_8 resulted in a lower fraction of tolerant individuals than did injections with the same quantity of the same antigens between the 44th and 73rd day ($P < 0.05$; comparison of groups I-d and I-e with I-f and I-g of Table I).

We next examined the maintenance of tolerance in animals injected with HA at birth and given chemically modified antigens up to the time when tolerance would have been lost in all animals had they not received cross-reacting antigens. To do this, animals were injected with HA at birth, and between the 44th and 248th day were given HA-D_8, HA-D_{35}, HA$(Ox)_8$, or HA$(Ox)_{38}$. Finally, on the 260th day they were given HA.^{131}I. A large proportion (50–100%) of these animals maintained tolerance (comparison with animals which received HA at birth but no cross-reacting antigens thereafter: $0.001 < P < 0.01$). The proportion of animals which maintained tolerance was similar in the groups treated with HA-D and HA(Ox) (Table II); there was no difference at the 5% level; ($0.50 < P < 0.70$). Further, HA-D_8 and HA-D_{35} were equally effective in maintaining tolerance ($0.10 < P < 0.20$), whereas HA$(Ox)_{38}$ was less effective than HA$(Ox)_8$ ($P = 0.012$).

We therefore conclude that tolerance can be prolonged by injecting a cross-reacting antigen, provided that this treatment is started within a critical period.

It appears also that different cross-reacting antigens may differ in their capacity to prolong tolerance.

Incidence and Development of Antibody Production in Tolerant Animals.— Having dealt so far with the effect of cross-reacting antigens on the maintenance of tolerance, we shall now turn to the response to HA.^{131}I of those animals in which prolongation of tolerance did not occur. The time at which the third phase of elimination occurred ($t_{0.1}$) was determined in these animals and in a control group. Animals in the control group received antigen at birth, no cross-

TABLE I

Effect of Cross-Reacting Antigens on the Maintenance of Tolerance

Animals were injected with 20 mg human albumin (HA) at birth, and were subsequently given two to four courses of injections of HA or of *p*-azo sulfonic acid derivatives of HA (HA-D_8, HA-D_{16}, and HA-D_{35}). A course consisted of three injections of 5 mg protein each, given at 48-hr intervals. These courses of injections were administered on the following days after birth: 44, 46, 48; 69, 71, 73; 94, 96, 98; and 119, 121, 123. Lightly iodinated HA (HA.^{131}I) was injected on day 135.

Group	Days after birth at which antigen (HA-D_n*) was injected				Fraction of animals which eliminated HA.^{131}I diphasically	$t_{0.1}$‡
	44, 46, 48	69, 71, 73	94, 96, 98	119, 121, 123		
						hr
I-a	HA-D_{35}	HA-D_{35}	HA-D_{35}	HA-D_{35}	6/6	—
I-b	HA-D_{16}	HA-D_{16}	HA-D_{16}	HA-D_{16}	6/6	—
I-c	HA-D_8	HA-D_8	HA-D_8	HA-D_8	8/9	120
I-d	HA-D_8	HA-D_8	Nil	Nil	12/12	—
I-e	HA	HA	Nil	Nil	5/5	—
I-f	Nil	Nil	HA-D_8	Ha-D_8	9/12	70,100,310
I-g	Nil	Nil	HA	HA	8/10	80,210

* n was 0, 8, 16, or 35.

‡ $t_{0.1}$ is the time in hours at which the circulating antigen is 0.1% of m_0; m_0 is found by extrapolating the metabolic phase to zero time.

reacting antigen thereafter, and HA.^{131}I between the 84th and the 295th day of life. A proportion of these animals lost tolerance.

The value for $t_{0.1}$ was shorter in the animals which had received cross-reacting antigen than in the control animals (Fig. 1). The rapid onset of immune elimination of HA^{131}I in the former group may be connected with antibodies formed against determinants of the cross-reacting antigens, and we shall next examine the formation of such antibodies. By agglutination of tanned erythrocytes coated with either HA or cross-reacting antigen (HA-D_8, HA-D_{35}, HA$(Ox)_8$, HA$(Ox)_{38}$), antibody could be detected in 48/103 or 52/103 animals, respectively. The incidence of responders was highest among animals immunized with HA$(Ox)_{38}$ and lowest among those which were given HA$(Ox)_8$. There was

no increase in the incidence of responding animals between the 4th and 9th course of injections (Table III). We followed the serum agglutination titer of all animals through nine courses of injections. Animals which had failed to form antibody after two courses of injections with HA-D_8 or HA$(Ox)_{38}$ remained unresponsive to the seven subsequent courses (Fig. 2).

During progressive immunization, we observed two types of changes of antibody quantity. The rate of increase in reciprocal titers of the 27 animals responding to HA(Ox) and HA-D was greatest after the initial two to four

TABLE II

The Elimination of Iodinated Human Albumin (HA.^{131}I) from the Circulation of Animals Injected at Birth with Human Albumin (HA) and Subsequently with Derivatives of HA

Rabbits were injected with 20 mg HA at birth, and were given nine courses of injections of derivatives of HA between day 44 and 248. A course consisted of three injections each of 5 mg protein given at 48-hr intervals. HA.^{131}I was injected intravenously on day 260.

Group	Hapten molecule attached to HA	Average number of hapten groups per molecule of HA	Fraction of animals which eliminated HA.^{131}I diphasically	$t_{0.1}$
				hr
II-a	*p*-azo sulfonic acid	8	5/10*	270, 260, 170, 220, 140
II-b		35	12/16‡	220, 450, 110, 100
II-c	Oxazolone	8	10/10§	—
II-d		38	6/12‖	250, 230, 160
II-e	None	0	5/5¶	—

* and ‡ compared with § and ‖ $0.50 < P < 0.70$.

* compared with ‡ $0.10 < P < 0.20$.

§ compared with ‖ $0.001 < P < 0.01$.

¶ compared with*+‡+§+‖ $0.10 < P < 0.20$; with * $P < 0.05$; with ‡ $0.10 < P < 0.20$; with § $P > 0.50$; with ‖ $P < 0.05$.

courses, and decreased after subsequent immunization. In 16/27 animals, the quantity of agglutinating antibody reached a peak level and then decreased; in 6 of these 16 animals this resulted in loss of detectable antibody. In the remaining 11 responding animals (11/27) a constant level of circulating antibody was reached and maintained. This constant level was observed in most responders to HA-D_8 (5/6), and in one-third of responders to HA-D_{35} (3/10) and to HA-$(Ox)_{38}$ (3/10), but not in the one responder to HA $(Ox)_8$.

Reactivity and Specificity of Antibody.—The antibodies of most responders (26/27) agglutinated tanned erythrocytes coated with native HA. This reactivity was only found in sera which agglutinated tanned erythrocytes coated with cross-reacting antigen. The titer detected with HA was always smaller

than that detected with modified antigen. This difference was small in sera from animals injected with HA-D_8 (average ratio HA-D_8/HA = 1.4) and in sera from the one animal which responded to HA$(Ox)_8$ (average ratio HA$(Ox)_8$/HA

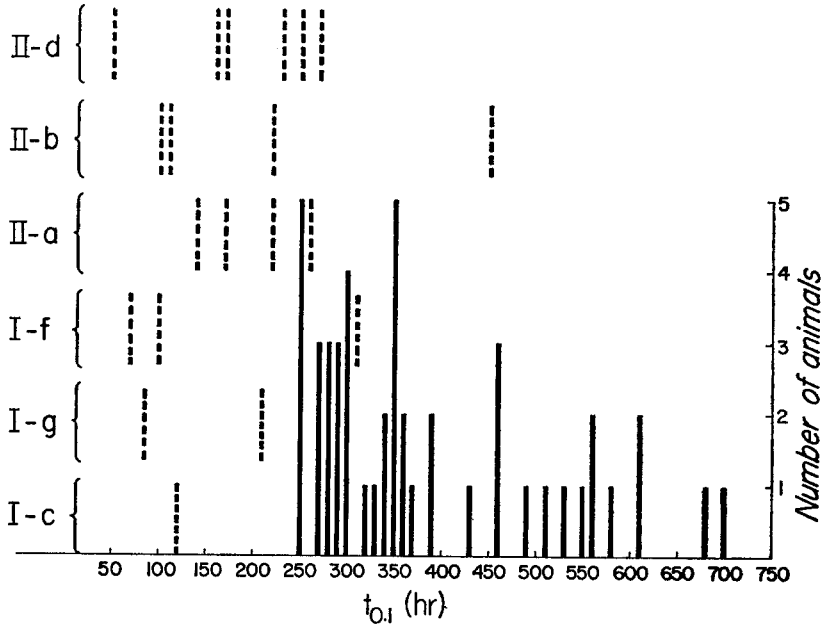

FIG. 1. Distribution of the periods of time ($_{0.1}$) required for the disappearance of iodinated human albumin (HA.^{131}I) from the circulation of animals which eliminated HA.^{131}I in a triphasic manner. Animals were injected at birth with 20 mg HA. All animals were later given 5.24 mg HA.^{131}I per kg of body weight intravenously. Number on left hand side of figure refers to the number of the table which also describes these animals. The accompanying letter designates the group within the table. Number and letter refer only to the broken lines opposite them. The solid lines designate animals injected with HA at birth which received HA.^{131}I and no other injections in later life. Group II-d rabbits were given nine courses of injections of HA$(Ox)_{38}$ between day 44 and 248. Groups II-a and II-b were given nine courses of injections of HA-D_8 and HA-D_{35} respectively between day 44 and 248. All animals of groups II-d, II-b, and II-a were injected with HA.^{131}I at age 260 days. Animals of group I-c were given four courses of injections of HA-D_8 between day 44 and 123. Animals of groups I-f and I-g were given two courses of injections of HA-D_8 and HA respectively between day 94 and 123. All animals of groups I-f, I-g, and I-c were injected with HA.^{131}I on day 135.

= 1.8); it was much larger in sera from animals injected with HA-D_{35} (average ratio HA-D_{35}/HA = 6.1) and HA$(Ox)_{38}$ average ratio HA$(Ox)_{38}$/HA = 6.2). In 2/16 responders to HA-D and in 6/9 responders to HA$(Ox)_{38}$ antibodies reactive with modified antigen could be detected in sera in which antibodies could no longer be detected with native HA.

Antibody specificity of sera obtained after each course of immunization was

examined by the agglutination-inhibition test, employing tanned erythrocytes coated with the immunizing antigen. The agglutinating capacity of sera from animals injected with HA-D_8 or HA$(Ox)_8$ was completely inhibited by HA-D_8 or HA$(Ox)_8$, respectively, and was reduced one to two dilution steps by native HA (8). There was no consistent difference in this latter property between anti-

TABLE III

Incidence of Agglutinating Antibody in the Sera of Animals Rendered Tolerant to Human Albumin and Subsequently Injected with Chemical Derivatives of Human Albumin

Rabbits were injected with 20 mg human albumin (HA) at birth and were later injected with azo human albumin (HA-D) or oxazolonated human albumin HA(Ox) according to the following schedule. Injections with the chemical derivatives of HA were given on day 44, 46, 48; 69, 71, 73; 94, 96, 98; 119, 121, 123; 144, 146, 148; 169, 171, 173; 194, 196, 198; 219, 221, 223 and 244, 246, 248. Bleedings were taken on days 54, 59; 78, 84; 103, 109; 128, 134; 153, 159; 178, 184; 203, 209; 228, 234; 253, 259, and the agglutinating titer was determined with tanned erythrocytes sensitized with the antigen employed for immunization.

Human albumin derivative (HA-X*) used in immunizations	Average No. of hapten groups per HA molecule	Fraction of responders‡ (period in days during which animals were immunized, and antigen (HA, HA-X*) with which tanned red cells were sensitized)					
		73 days		123 days		248 days	
		HA-X	HA	HA-X	HA	HA-X	HA
HA-D	8	10/58	8/58	7/23	6/23	6/10	6/10
	16	2/12	0/12	2/10	0/10	nd	nd
	35	14/37	6/37	32/48	32/48	4/8	4/8
HA(Ox)	8	1/40	1/40	1/10	1/10	1/10	1/10
	38	18/20	11/20	10/12	9/12	10/12	9/12
Totals.....	.....	45/167	26/167	52/103	48/103	21/40	20/40

nd, not done.

* HA.X is HA-D_8, HA-D_{16}, HA-D_{35}, HA$(Ox)_8$, or HA$(Ox)_{38}$ depending on the antigen used for immunization.

‡ Animals considered as antibody producers if antibody is found in any of the two bleedings taken after each course of injections.

bodies obtained after two and nine courses. After two courses of injections the agglutinating capacity of sera from animals immunized with HA$(Ox)_{38}$ or HA-D_{35} was not reduced by HA (7, 8). This was also found with sera from animals given further courses of injections of HA$(Ox)_{38}$. However, in the sera of animals given four to nine courses of injections of HA-D_{35} some degree of inhibition was exercised by HA (1–5 dilution steps in 3/4 animals).

The specificity of the antibody fraction reactive with HA, was examined with tanned cells coated with the native protein. The agglutinating capacity of antibodies produced after two courses of immunization with HA-D_8 could be com-

pletely inhibited by HA-D_8 and by HA, but more HA than HA-D_8 was required for a given degree of inhibition (8). This changed in the course of continued immunization, so that sera obtained after four to nine courses of injections, were inhibited to a similar extent by HA and HA-D_8 (Fig. 3., rabbit 30-74 A).

Sera obtained from animals given two courses of injections of HA-D_{35} reacted only weakly with tanned erythrocytes coated with HA (mean log titer 2.0 ± 0.3), so that agglutination-inhibition tests could not be carried out. However, further immunizations with HA-D_{35} produced antibodies which reacted more strongly with tanned erythrocytes coated with HA (mean log titer

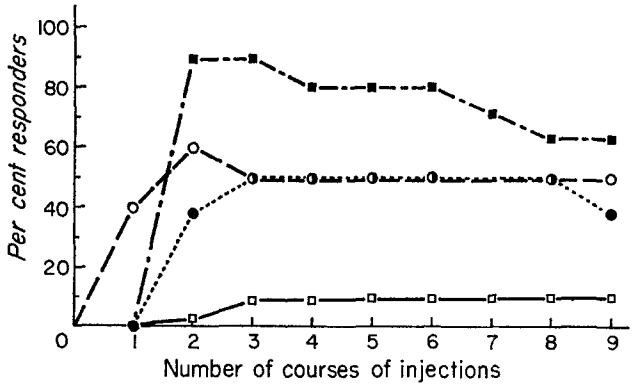

FIG. 2. Response of rabbits which were injected with human albumin (HA) at birth and given nine courses of injections of chemically modified derivatives of HA during adult life. Each point on the graph refers to the percentage of animals which made antibody after a particular course of injections (based on the agglutination of tanned cells coated with the immunizing antigen). Animals which did not respond at this stage are not included even though they may have responded at a different stage. ○, rabbits immunized with HA-D_8; □, rabbits immunized with HA$(Ox)_8$; ●, rabbits immunized with HA-D_{35}; and ■ rabbits immunized with HA$(Ox)_{38}$.

3.5 ± 0.6). The agglutinating capacity of all these antibodies was more effectively inhibited by HA-D_{35} than by HA. However, the relative quantities of HA and HA-D_{35} required for comparable depression of titers varied between one and eight for sera from different individuals (curves in the lower half of Fig. 4).

The HA-reactive antibody obtained after two courses of immunization with HA$(Ox)_{38}$, differed from the HA-D antibodies, in being more reactive with HA than with the chemically modified antigen (7). In some animals continued immunization with HA$(Ox)_{38}$ did not bring about a substantial increase of titer and led to the continued production of this type of antibody (Fig. 3, rabbit 21–38 G). In other animals the properties of the antibody underwent a marked progressive change, in that agglutinating capacity was equally effectively inhibited by both antigens, or was more effectively inhibited by HA$(Ox)_{38}$ than

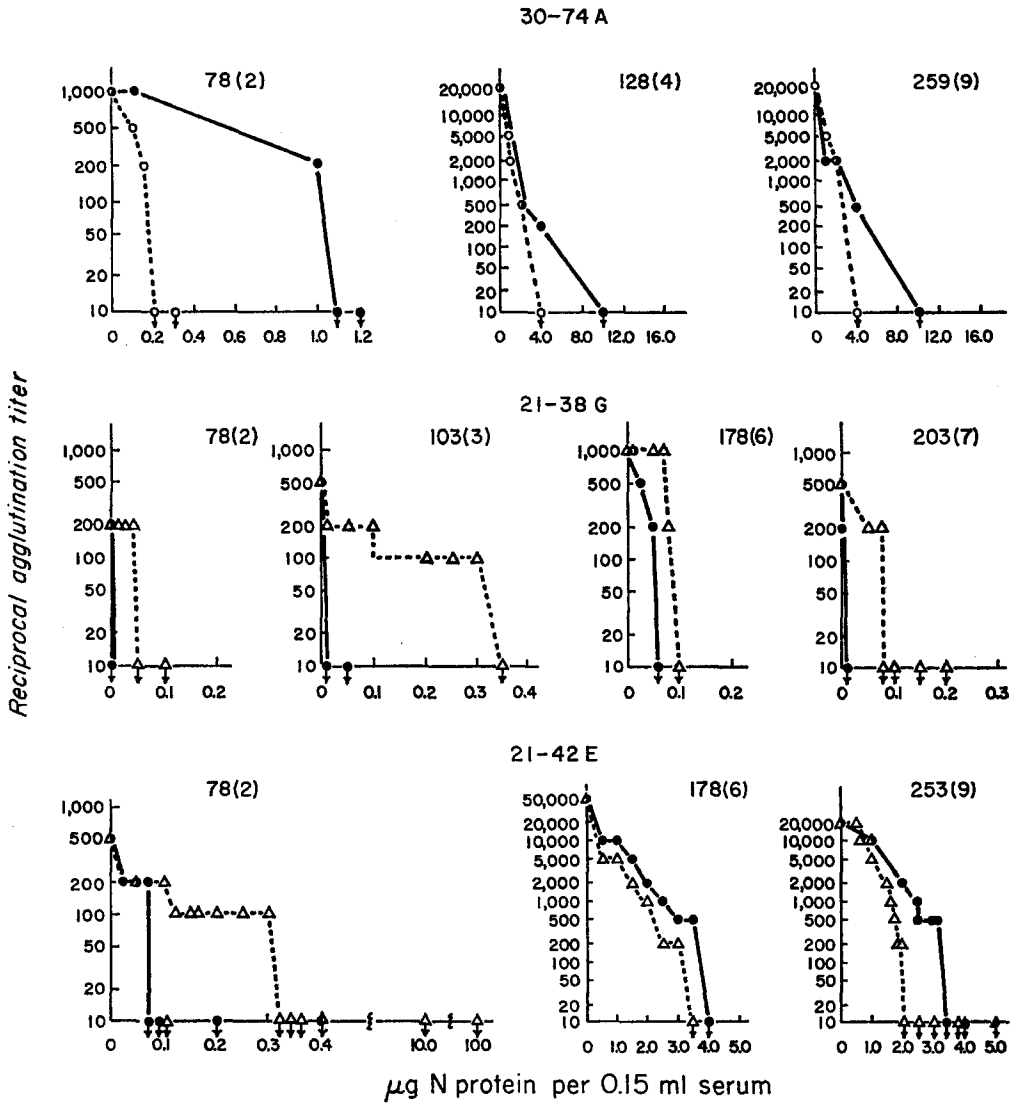

FIG. 3. The antibody specificity of sera obtained from animals injected at birth with human albumin (HA) and subsequently with chemically modified derivatives of HA. These curves represent three types of specificity changes found in sera from rabbits immunized with HA-D and HA(Ox). Agglutination tests were carried out with tanned cells coated with HA. The capacities of HA, HA-D_8, or $HA(Ox)_{28}$ to inhibit agglutination were examined. Number above each row of graphs indicates designation of rabbit. Number above each graph gives the age, in days, of the animal when the serum was obtained. Numbers in brackets indicate the number of courses of injections an animal received before the serum was obtained. ●, reciprocal titer after inhibition with HA; ○, reciprocal titer after inhibition with HA-D_8; and △, reciprocal titer after inhibition with $HA(Ox)_{28}$.

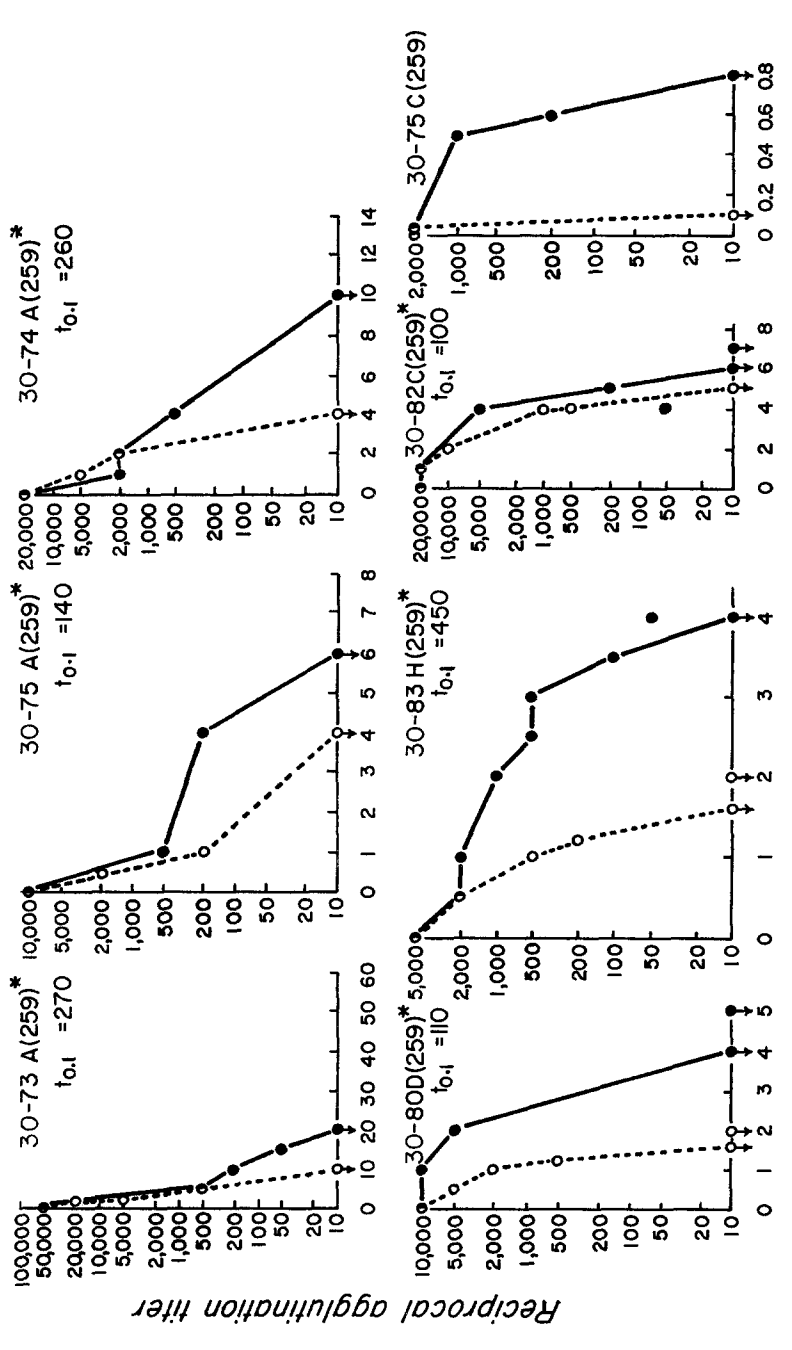

FIG. 4. The antibody specificity of sera obtained from animals injected with human albumin (HA) at birth and subsequently given nine courses of injections of HA-D. Agglutination tests were carried out with tanned cells coated with HA. The capacity of HA or HA-D to inhibit the agglutination reaction was determined. Number above each graph indicates designation of rabbit. Number in brackets gives the age, in days, of the animal when serum was obtained. Asterisks (*) identify animals which eliminated HA.^{131}I in a triphasic manner. HA.^{131}I was given after the ninth course of injections of HA-D. Curves in the upper part of the figure were obtained with sera from animals immunized with HA-D_8, whereas curves in the lower part represent agglutination-inhibition tests with sera from animals immunized with HA-D_{35}. ●, reciprocal titer after inhibition with HA; and ○, reciprocal titer after inhibition with HA-D.

by HA. In this type of animal, the antibody titer increased during continued immunization (Fig. 3, rabbit 21–42 E).

We have thus detected a progressive change in the properties of a *fraction* of the total antibody which reacts with tanned cells coated with HA. This change was not found in the sera of all the animals which responded to protein derivatives. Where it *was* observed, we also found triphasic elimination at the end of nine courses of immunization (Fig. 4). However, one animal did eliminate HA.^{131}I triphasically, though the HA antibody after nine courses was of a type characteristic of the early antibody of other responders (Fig. 4, rabbit 30–83 H). In this animal the onset of immune elimination occurred 18 days after the injection of HA.^{131}I ($t_{0.1}$ = 450 hr). This value of $t_{0.1}$ was similar to that of animals which were injected at birth but were not given cross-reacting antigens (Fig. 1). In contrast, the immune phase of elimination of all the other animals, which received antigen at birth and cross-reacting antigen thereafter, started very much earlier than in animals which were injected at birth, received no cross-reacting antigens, and were tested at the same time as the animals injected with cross-reacting antigens (Fig. 1).

Sensitivity of Antibody to Treatment with 2-Mercaptoethanol.—The agglutinating capacity of sera obtained from 13/31 animals which responded to two courses of immunization with HA-D, could be completely inhibited by pretreatment with mercaptoethanol (8). Antibodies obtained after three to nine courses of injections were mercaptoethanol insensitive in all but one serum. The mercaptoethanol-sensitive serum was obtained from a rabbit after seven courses of injections of HA-D_{35}, and this particular animal showed no detectable antibody after the eighth and ninth course of injections. Another animal, which made antibody initially but not finally, lost its capacity to make antibody after the third course of injection of HA-D_8. The agglutinating capacity of the serum obtained after the second course was mercaptoethanol sensitive.

The agglutinating capacity of sera obtained from 2/11 animals which responded to two courses of immunization with HA$(Ox)_{38}$, could be completely inhibited by pretreatment with mercaptoethanol. Antibodies obtained in all animals after the third and fourth courses of injections, were insensitive to mercaptoethanol.

DISCUSSION

Acquired immunological tolerance may be considered as a regulatory mechanism, restricting the antibody response to determinants which are not identical with or similar to autologous macromolecules. Antibodies can only be elicited by determinants to which tolerance has not been induced unless tolerance is circumvented. Autoantibody formation may sometimes be due to such circumvention, and this can be studied by rendering animals tolerant to one antigen and immunizing them subsequently with a cross-reacting antigen. The first

studies of this type showed that an antibody could be obtained which possessed reactivity towards the tolerance-inducing antigen (1). The connection between this reactivity and termination of tolerance was formulated by Weigle (13); it may perhaps be described better as "circumvention" since it may be attributable to antibodies which are directed against determinants which are *not* identical in the tolerance-inducing and in the immunizing macromolecule (8). It appeared that there may also be other effects of the cross-reacting antigen in that it might prolong the duration of tolerance. This would not affect natural tolerance, but might be important when tolerance has been induced experimentally. An analysis of this effect is necessary if the regulatory consequences of natural tolerance (14, 15) are to be elucidated on the basis of model experiments. Most laboratory experiments differ in one important respect from the conditions which prevail in naturally acquired tolerance: in the laboratory experiment antigen is provided discontinuously, in the natural system it is provided by synthesis and hence continuously. As a consequence of the discontinuous supply of tolerance-inducing macromolecules, tolerance is ultimately lost and a dynamic situation must prevail prior to this. In spite of this complication, the discontinuous supply of tolerance-inducing macromolecules has important advantages since this avoids removal of a fraction of antibody by combination with circulating tolerance-inducing macromolecules and hence permits detection of all antibodies. However, it is necessary to differentiate between the extent to which cross-reacting antigens prolong the tolerant state, and the extent to which they circumvent the tolerant state by inducing formation of antibodies with cross-reactivity towards the tolerance-inducing macromolecule. Circumvention may be attributed to antibodies which are evoked by determinants to which the animal is not tolerant. These antibodies are presumably the product of cells that cannot respond to the tolerance-inducing macromolecules.

The question arises whether, in addition, the cross-reacting antigen preserves the inability of cells to respond to the tolerance-inducing antigen. To determine this, we have employed the mode of elimination of the tolerance-inducing macromolecule as an operational criterion of immune responsiveness. By this means we have compared the responses of animals injected at birth and then immunized with cross-reacting antigen with those of animals that were injected at birth but not further immunized. By this criterion, it has been clearly established that cross-reacting antigens can prolong the duration of tolerance (Table II). This was also found when the incidence in the immunized group (HA-D_8, HA-D_{35}, HA$(Ox)_8$, but not HA$(Ox)_{38}$) of animals which made agglutinating antibody, was compared with the incidence of nonimmunized animals which maintained tolerance as judged by elimination tests.

Tolerance can be prolonged indefinitely in all animals if instead of the cross-reacting antigen, tolerance-inducing antigen is given during later life (reference 16, and Table II). A comparison of groups of animals given, during adult life,

cross-reacting and tolerance-inducing antigen, respectively, reveals that tolerance is better maintained by the antigen which was given at birth than by a cross-reacting antigen (Table II). To identify this difference with antigen-induced circumvention of tolerance would obviously be erroneous, since it is, in fact, due to differences in the effectiveness with which tolerance is prolonged.

We shall now turn to a consideration of factors which appeared to be related to the loss of tolerance in a proportion of the neonatally injected and immunized animals. The reactivity towards HA.^{131}I of the antibody responders was distinct from that of nonimmunized neonatally injected animals. Animals which did respond to cross-reacting antigen responded more promptly to HA.^{131}I than did animals which had not received cross-reacting antigen, that is, the third phase of immune elimination appeared earlier.

The properties of protein-specific antibodies from neonatally injected and subsequently immunized animals underwent changes in the course of prolonged immunization. Different changes were observed in animals immunized with HA-D_8, HA-D_{35}, and HA$(Ox)_{38}$. Animals which were given azo derivatives produced antibody which became progressively better adapted to HA (Fig. 3). The situation was somewhat more complex in tolerant animals which were immunized with HA$(Ox)_{38}$. In the initial response to this antigen all the responders made a heteroclitic antibody (17) which was better adapted to HA than to HA$(Ox)_{38}$ (7). This kind of antibody continued to be formed in some animals during the course of further injections, while in other animals it became equally or better adapted to HA$(Ox)_{38}$ (Fig. 3). Therefore two distinct families of antibody have to be taken into account. The presence of heteroclitic antibody may obscure the specificity changes which are clearly seen in the response to HA-D.

Our data on specificity changes do not allow us to decide whether this change is due to antibodies which are adapted to the same site and to a progressively wider area surrounding the site (5, 18), or whether there is synthesis of new types of antibodies against determinants which were originally excluded from immunogenicity, but can induce antibody formation in a population of cells which appears as the animal becomes older. We can obtain some insight into this problem from the changes in antibody quantity.

In the course of immunization most neonatally injected animals reached a peak titer by the fourth course of immunization. After further immunization a constant titer was maintained or a decrease in titer occurred, sometimes to the point at which antibody could not be detected. This was previously observed (2, 19) and has been described as a "return of the tolerant state," (19). In actual fact it may represent an instance of feedback inhibition (20–23). In an animal which is not tolerant to a cross-reacting antigen, a large number of determinants are immunogenic and antibody directed against some of these may have induced feedback inhibition when other determinants may have not yet induced peak titers. In animals which are tolerant to most determinants of

an antigen, the number of determinants is much more restricted and feedback inhibition, therefore, might be more readily detected. The above mentioned inhibition was observed in six animals immunized with $HA(Ox)_{38}$, two animals immunized with $HA\text{-}D_{35}$, and one animal immunized with $HA\text{-}D_{8}$. In most of these animals (8/9) the antibodies detected with cells coated with the modified antigen and with HA did not disappear at the same time. As a rule the antibody directed to HA disappeared first (7/9 animals). It seems likely that this asynchronous disappearance of antibodies is not due to the relative sensitivity of the method by which they are detected and is attributable to their production by different cell populations. This phenomenon was only observed during the period in which most animals would have lost tolerance if they had not been given the cross-reacting antigen. The loss of tolerance of immunized animals may thus be attributable to cell populations which recovered from tolerance or which appeared during this period by differentiation from stem cells, and were sensitive to determinants which are very similar in tolerance-inducing and immunizing macromolecules.

Among animals immunized with HA-D, feedback inhibition may perhaps be connected with the mercaptoethanol sensitivity of the antibody. Antibodies formed early by tolerant animals and also by normal animals were sensitive to treatment with 2-mercaptoethanol (8); antibodies formed after repeated injections were resistant to 2-mercaptoethanol. The loss of the ability to produce agglutinating antibody was preceded by the continued presence or reappearance of mercaptoethanol-sensitive antibody. Whether the antibody is implicated in the loss of the ability to continue producing antibody is not clear, but it has been shown that antibodies which are sensitive to 2-mercaptoethanol can inhibit the immune response (21, 24).

We have suggested that prolongation of tolerance did not occur when antigen-sensitive cells appeared and responded to determinants which were similar in tolerance-inducing and immunizing antigen. We shall examine this now in terms of the relation between circulating antibody and the interval between injection and immune elimination of HA.^{131}I. Both the quantity and specificity of the antibody elicited by cross-reacting antigen, appeared to affect the mode of elimination of HA.^{131}I. All animals which showed high levels of agglutinating antibodies appeared to eliminate HA.^{131}I triphasically, but so did some animals whose sera showed no prior reactivity with native antigen. In both these groups the onset of the immune phase was earlier than in animals which were injected at birth, but were not immunized subsequently (Fig. 1). Clearly, the modified antigens had "primed" cells of the responding, neonatally injected animals, even if this had not resulted in prior antibody formation.

The specificity of the HA-antibody was not identical in all responding animals. If this reactivity could be inhibited by HA as effectively as by modified HA then the animal which produced such antibody went on to lose tolerance

(Fig. 4). Thus the presence of antibodies which were well adapted to HA appeared to indicate that an animal would go on to lose tolerance. The exceptional loss of tolerance in one animal (Fig. 4, rabbit 30–83 H) that made antibody which was poorly adapted to HA probably involved a different mechanism since the inception of immune elimination occurred very late; $t_{0.1}$ = 450 hr. This value of $t_{0.1}$ is comparable to that of animals which lost tolerance spontaneously and had not been injected with cross-reacting antigen.

In animals, immunized with $HA(Ox)_{38}$, the production of heteroclitic antibodies was not related to loss of tolerance. Triphasic elimination was only observed if antibody evolved which was similar in specificity to that described for animals immunized with HA-D.

We have thus seen that cross-reacting antigen can prolong the duration of tolerance. Some animals do not show this effect and form antibodies which may be produced by cells which arise after attrition of the neonatally induced tolerance and which are sensitive to determinants of the tolerance-inducing antigen.

SUMMARY

Rabbits were rendered tolerant to human albumin (HA) and were then injected with azo and oxazolonated derivatives of human albumin. These injections were continued to a time at which all animals would have lost tolerance if they had not been injected. Injection of cross-reacting antigens prolonged the duration of tolerance, as judged by the mode of elimination of lightly iodinated human albumin (HA.^{131}I). Different derivatives of HA differed in their capacity to prolong tolerance.

Those neonatally injected rabbits which were immunized with cross-reacting antigens and lost tolerance, responded much more promptly to HA.^{131}I than animals which were not immunized. Animals immunized with cross-reacting antigen which went on to eliminate HA.^{131}I triphasically, usually had responded earlier by making antibodies. These antibodies contained a fraction which was reactive with HA, and which was usually equally well adapted to determinants on HA and on the cross-reacting antigen.

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