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Degeneracy of the Immune Response to Sheep Red Cells

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Summary. We found that some mice immunized with sheep red blood cells (SRBC) made antibodies that agglutinated horse RBC (HRBC). Absorption studies indicated that the cross-reacting antibodies were highly avid. They appeared either late in a primary response, in secondary responses, or after multiple immunizations. Thus, the antibody response to SRBC loses specificity with time and is a further example of the degeneracy of the immune response. The similarities between the system we described and previously studied examples of degeneracy suggest that their bases may be the same; reactions of high affinity antibodies with related determinants. This interpretation was strengthened by the observation that antibodies made by mice which had recently escaped from tolerance were particularly deficient in cross-reacting subsets. The possible pitfalls of using hyperimmune serum in tests requiring high degrees of immunological specificity were emphasized.

INTRODUCTION

It has been shown that as an anti-hapten immune response progresses the antibodies made exhibit increasing cross-reactions with related as well as unrelated haptens (Little and Eisen, 1969; Little, Border and Freidin, 1969; Underdown and Eisen, 1971). Such cross-reactions are even more prominent in secondary responses. This loss of specificity of the immune response with time has been referred to as 'degeneracy' (Little and Eisen, 1969). Other examples of the degeneracy of the immune response include a decline in carrier specificity (secondary anti-hapten responses can be elicited with the hapten on a carrier other than the one used for immunization) (Steiner and Eisen, 1967; Paul, Siskind, Benacerraf and Ovary, 1967) as well as increased non-carrier dependent responsiveness to related haptens (Steiner and Eisen, 1967). This second phenomena is similar to 'original antigenic sin' (Fazekas de St Groth and Webster, 1966) which has been considered to be another example of degeneracy (Little and Eisen, 1969).

We have examined the ability of antibodies made in response to immunization with sheep red blood cells (SRBC) to agglutinate horse RBC (HRBC). Previous studies have failed to detect significant cross-reactions between these two antigens (Cunningham, 1966; Radovich and Talmage, 1968, Möller and Sjöberg, 1970; Eidinger *et al.*, 1971). Indeed, many workers have used this antigen pair in experiments where one antigen served as a specificity control for the other (Radovich and Talmage, 1968; Miller and

Mitchell, 1968; Mitchell and Miller, 1968; Haskill, 1969; Albright, Omer and Deitchman, 1970; Gershon and Kondo, 1970; Miller and Mitchell, 1970; Möller and Sjöberg, 1970; Eidinger *et al.*, 1971; O'Toole and Davies, 1971).

We have found that the immune response to SRBC exhibits a form of degeneracy, in that antibodies made late in a primary response, in a secondary response or after hyper-immunization may show extensive cross-reactions with HRBC. The possible relationship of these cross-reactions to other examples of degeneracy are discussed.

MATERIALS AND METHODS

Mice

Male or female CBA mice were used in these experiments. They were either strain CBA/H from our own colony or strain CBA/J from Jackson Laboratories, Bar Harbor, Maine. All experiments were controlled for sex or strain of mouse.

Thymectomy

Thymectomies were performed on adult mice, 7–8 weeks of age, under light ether anaesthesia following the technique of Miller (1960). At the termination of experiments all mice were autopsied and thymic remnants were searched for. None were found in any animals reported in these experiments.

Irradiation

A mid-axis dose of 850 R was delivered from a Siemans 250 KV machine at a dose rate of 85 R/minute.

Cell suspensions

Bone marrow cell suspensions were prepared by washing out the femurs of adult syngeneic mice with cold sterile tissue culture medium 199. Thymus cell suspensions were prepared by gently teasing thymuses of syngeneic weanling (4–5 weeks of age) mice between sterile glass slides in cold medium 199. They were filtered through gauze and washed before injection. Counts of viable cells were made in a haemocytometer using the trypan blue dye exclusion method. The cells were injected intravenously via the tail vein.

Preparation of chimeras

Irradiation was performed 1 week after thymectomy and the mice were reconstituted with 5×10^6 syngeneic bone marrow cells on the same day. At least 30 days were allowed to elapse before these thymus-deprived chimeras were immunized. When thymocyte reconstitution was done, it was done on the same day as the initial immunization.

Red blood cells

These were obtained in Alsever's solution washed three times before use and injected intraperitoneally in a final volume of 0·2 ml.

Bleeding

Bleeding was done with capillary pipettes placed in the retro-orbital sinus. Serum was separated and used for titration within 24 hours. Individual mice were ear-marked so that each could be followed serially.

Absorptions

Absorptions were performed by incubating 2 parts of serum with 1 part of packed red blood cells at 37° for 1 hour. After incubation the cells were separated from the serum by centrifugation at 500 *g* for 10 minutes. Since HRBC are about 15 per cent larger than SRBC this means we probably used slightly more SRBC antigen per absorption.

Titrations

Sera were individually titrated by the microhaemagglutination technique described by Sever (1962). The titres were expressed as the $\log_2$ of the last well showing macroscopic agglutination. Thus, if the undiluted serum showed no agglutination the titre is expressed as $\bar{1}$. A titre of 0 means agglutination occurred with whole serum but not at a 1:2 dilution in isotonic saline. After the results had been recorded (all results were read separately by two observers in a 'blind' fashion) the red cells were resuspended by gentle tapping of the plates and 0·025 ml of 0·1 M 2-mercaptoethanol (ME) was added to each well. The cells were allowed to resettle at room temperature and end-points were read as before. These titres were taken to represent ME resistant (MER) antibody. This method of ME inactivation has been studied at some length and has been shown to produce the same results as more standard techniques (Scott and Gershon, 1970). It was used in these studies in order to minimize the blood loss of experimental animals. MER antibody may be considered roughly equivalent to 7S antibody under ordinary circumstances (Adler, 1965).

Statistical analysis

Student's *t*-test was the method used in all statistical analyses.

RESULTS

I. EFFECT OF IMMUNIZATION SCHEDULE ON THE PRODUCTION OF CROSS-REACTING ANTIBODIES

This experiment demonstrates the timing of the appearance of HRBC agglutinating antibodies after SRBC immunization. Two groups of eight mice were immunized with 5×10^8 SRBC. One group received the same dose every 2 weeks while the other was reinoculated only once, at 14 weeks after the first dose. All mice were bled every 2 weeks and their sera were titrated against SRBC and HRBC. The mean anti-SRBC titre and the individual HRBC titres of these mice are plotted in Fig. 1. The primary response to SRBC peaked at 4 weeks and then slowly declined while multiple inoculations resulted in a slow continuous rise in anti-SRBC haemagglutinins. It is interesting that while the anti-SRBC titres were declining with time in the primary response, the anti-HRBC titres (of those mice that made cross-reacting antibodies) showed the opposite trend so that the anti-HRBC titres of the three responding mice (of eight) were at their peak 14 weeks after immunization with SRBC at a time when their anti-SRBC titres were at their nadir.

Mice receiving multiple SRBC injections also had peak anti-HRBC titres at this time and considerably more of these mice made cross-reacting antibody (7/7) than did the singly injected mice, but when these were inoculated a second time their anti-HRBC titres rose rapidly to reach similar levels both in mean titre and numbers of responders to those of the multiply inoculated group.

This experiment demonstrates that: 1. More anti-HRBC antibody was made in the

secondary anti-SRBC response than in the primary response. 2. The cross-reacting antibody that occurred in the primary response occurred late. 3. The titre of cross-reacting antibody was not necessarily related to the titre of the anti-SRBC antibody.

During the past year we have routinely titred sera from SRBC-inoculated mice against HRBC and these three points have been continually substantiated.

II. EFFECTS OF CROSS-ABSORPTION ON CROSS-REACTION ANTIBODIES

All the mice from the previous experiment were exsanguinated at 18 weeks and their sera were absorbed with both SRBC and HRBC. The effects of absorption on the haemag-

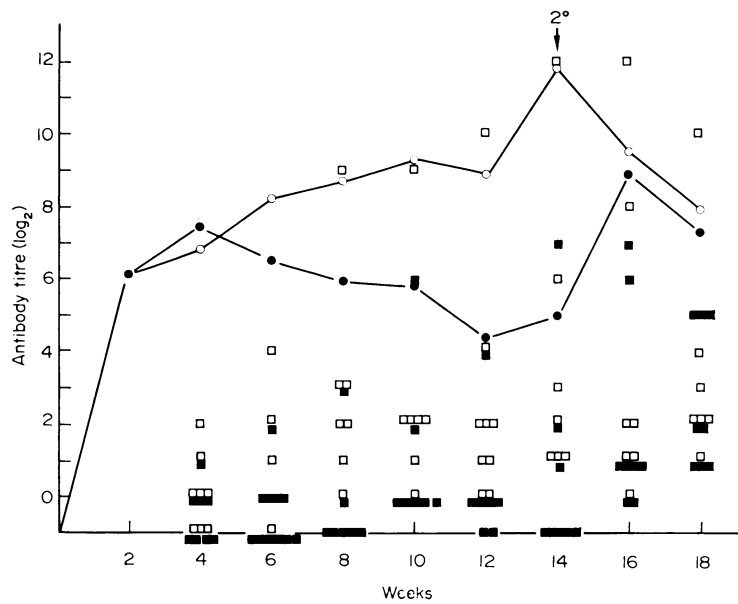

FIG. 1. Anti-SRBC (circles) and anti-HRBC (squares) titres of mice immunized with SRBC. Open symbols are titres of mice immunized every 2 weeks. Closed symbols are those of mice immunized on day 0 and given a second immunization at 14 weeks.

glutinating antibody titres are presented in Table 1. We have selected four sera from each of the two groups (multiple inoculations and two inoculations) for illustration; two from each with good cross-reactions and two with poor cross-reactions. The individual titres of the mice at 4, 14, and 18 weeks are given in the first three columns. The 18-week sera were diluted in M199 prior to absorption and the titres after dilution are given in the fourth column. The results of four consecutive absorptions are given in the remaining columns.

The following points emerge: 1. The anti-HRBC antibodies were preferentially absorbed by both SRBC and HRBC. This is most strikingly seen when the anti-HRBC titre is greater than the anti-SRBC titre, as was the case with the sera from mice numbers 4 and 7. The mean titre (log_2) of these two sera was 4·5 versus SRBC and 6·5 versus HRBC prior to absorption. After one absorption with SRBC the mean titre against SRBC was unchanged while the mean anti-HRBC was 1, a fall of 5·5. 2. SRBC and HRBC absorbed

TABLE 1

EFFECT OF DILUTION AND CROSS-ABSORPTION ON CROSS-REACTING ANTIBODIES MADE BY THE MICE WHOSE TITRES ARE REPORTED IN FIG. 1

Mice	Mouse No.	Anti-SRBC(S) and HRBC(H) antibody titres after: 4 weeks		14 weeks		18 weeks		Dilution		One absorption				Two absorptions				Three absorptions		Four absorptions	
										With SRBC		With HRBC		With SRBC		With HRBC		With SRBC	With HRBC	With SRBC	With HRBC
		S	H	S	H	S	H	S	H	S	H	S	H	S	H	S		S	S	S	S
Two SRBC inoculations (0+14 weeks)	1	6	1	4	1	8	2	3	1	2	0	4	0	1	0	3		0	4	0	4
	2	6	1	5	1	8	1	5	0	5	0	5	0	3	0	5		0	5	0	5
	3	6	3	5	7	7	5	4	2	2	0	3	0	1	0	3		0	3	0	3
	4	6	1	4	1	7	5	4	5	5	0	5	1	5	0	5		1	5	0	5
	Mean	6	0	4·5	1	7·5	3·3	4	2	3·5	0	4·3	0·3	2·5	0	4		0·3	4·3	0	4·3
Multiple SRBC inoculations (every 2 weeks)	5	10	3	12	1	8	2	5	2	3	0	4	0	3	0	5		0	5	0	5
	6	10	3	12	6	8	4	6	5	4	2	5	2	3	0	5		3	5	3	5
	7	8	9	12	12	6	10	5	8	4	2	5	3	3	0	4		3	5	2	5
	8	7	1	13	1	8	1	5	1	5	0	5	0	4	0	4		3	5	1	5
	Mean	8·8	4	12·3	5	7·5	4·3	5·3	4	4	1	4·8	1·3	3·3	0	4·5		2·3	5·0	1·5	5

the anti-HRBC equally well. 3. Absorption with HRBC did not significantly affect the anti-SRBC titres. 4. The sera of multiply-inoculated mice contained more anti-SRBC antibodies that were difficult to absorb than did the sera of mice inoculated only twice.

The four points made above have all been substantiated in multiple experiments. In them we have used a variety of multiple SRBC inoculation schedules, all of which result in the production of substantial cross-reactions. Furthermore we have found significant differences between strains in the production of the cross-reacting antibody. For example, our CBA/H mice make both more anti-SRBC antibody and more anti-HRBC antibody than do the CBA/J mice under similar conditions of stimulation. This point is illustrated in the following paper (Gershon and Kondo, 1972).

III. CROSS-ABSORPTION OF ANTIBODIES MADE EARLY IN THE PRIMARY RESPONSE TO SRBC, HRBC, OR BOTH

The preceeding cross-absorption studies were all done on sera of mice given multiple SRBC inoculations. Table 2 gives the results of cross-absorption studies on pooled sera

TABLE 2

EFFECT OF CROSS-ABSORPTION ON ANTIBODIES MADE 2 WEEKS AFTER PRIMARY ANTIGENIC STIMULATION

Mice immunized with:	Anti-SRBC(S) and HRBC(H) antibody titres after absorption with:					
	Nothing		SRBC		HRBC	
	S	H	S	H	S	H
HRBC	0	9	0	9	0	1
SRBC	6	0	0	0	6	0
HRBC+ SRBC	6	7	0	8	5	0

of mice given a single inoculation, of either 5×10^8 SRBC or HRBC; or a combination of the two. It can be seen that, under these conditions, there was no significant heterologous absorption.

IV. BLOCKING OF THE ABILITY OF HRBC TO ABSORB THE CROSS-REACTING ANTIBODY WITH NON-CROSSING-REACTING ANTI-HRBC ANTIBODY

Antibodies made in a primary response against HRBC differ from the anti-HRBC made in response to SRBC in that the former have no demonstrable affinity for SRBC. We made a further effort to see if we could demonstrate a relationship between these two antibody populations. Thus, we absorbed antiserum obtained 2 weeks after a primary HRBC immunization, with HRBC, and used the recovered cells to absorb the cross-reacting antibody made after multiple SRBC immunizations. The results (Table 3) show that HRBC incubated in primary anti-HRBC antibody (H-HRBC in Table 3) absorbed the cross-reacting antibody less well. Their absorbing capacity was diminished equally for both the cross-reacting and the primary anti-HRBC antibody. In addition these results illustrate, with another antiserum in which the anti-HRBC and anti-SRBC

titres are roughly equal, the preferential absorption of the cross-reacting antibodies by SRBC (see Table 1).

V. PRODUCTION OF ANTI-SRBC ANTIBODIES BY IMMUNIZATION WITH HRBC

We may now ask whether anti-SRBC antibodies can be produced by immunization with HRBC. The results from one experiment with multiple inoculations of 5×10^8

TABLE 3

EFFECT OF COATING HRBC WITH ANTI-HRBC ANTIBODIES ON THE ABILITY OF THE HRBC TO ABSORB CROSS-REACTING ANTI-SRBC ANTIBODIES

Antibody	Anti-SRBC(S) and HRBC(H) antibody titres after absorption with:							
	0		SRBC		HRBC*		H-HRBC†	
	S	H	S	H	S	H	S	H
Anti-SRBC (cross-reacting)	9	8	9	3	9	2	8	5
Anti-HRBC (non-cross-reacting)	0	8	0	8	0	2	0	5

* HRBC incubated in normal mouse serum or in primary anti-SRBC serum gave the same results.

† HRBC were incubated in anti-HRBC antibody before use in absorption studies. No significant elution of anti-HRBC antibodies occurred when these cells were incubated in normal mouse serum.

TABLE 4

ANTI-HRBC AND SRBC TITRES OF MICE IMMUNIZED WITH HRBC

No. of HRBC inoculations	Anti-HRBC titres (mean ± S.D.)	No. of mice with anti-SRBC titre >1	Mean anti-SRBC titres of responding mice
7	18·0 ± 1·3	2/7	4·5
6	15·3 ± 1·6	0/7	—
4	14·6 ± 1·0	0/5	—
3	12·4 ± 1·4	1/5	2
2	15·4 ± 1·0	3/5	3·3
1 (42 days)	9·0 ± 1·7	1/5	2
1 (14 days)	6·0 ± 0·6	0/5	—

HRBC into CBA/H mice (Table 4) show that anti-HRBC titres of these mice are much higher than the anti-SRBC titres of SRBC-inoculated mice and the amount of cross-reacting antibody is less.

VI. EFFECT OF TOLERANCE INDUCTION ON THE PRODUCTION OF CROSS-REACTING ANTIBODIES

In this experiment we looked for cross-reactions among the antibodies made after escape from tolerance. We made thymus-deprived, thymocyte-reconstituted CBA/H mice tolerant to SRBC by a previously described technique (Gershon and Kondo, 1970) and transferred their spleen cells into untreated thymus-deprived chimeras who were then

given an additional inoculum of normal thymocytes (Gershon and Kondo, 1971c) the object being to test the state of tolerance in B cells. A control group was treated the same way except that instead of getting spleen cells from tolerant chimeras, they got spleen cells from untreated chimeras. The mean anti-SRBC response and the anti-HRBC response of individual mice from these two groups is plotted in Fig. 2. It can be seen that the mice which got tolerant spleen cells made a diminished anti-SRBC primary response. Although the results are not given here, their MER antibody response was even more depressed (Gershon and Kondo, 1971c). After a second SRBC injection, at 3 weeks, their antibody titres recovered and after a third injection reached control levels. The number of these mice that made anti-HRBC antibodies was less than control however (7/12 vs 9/10) and

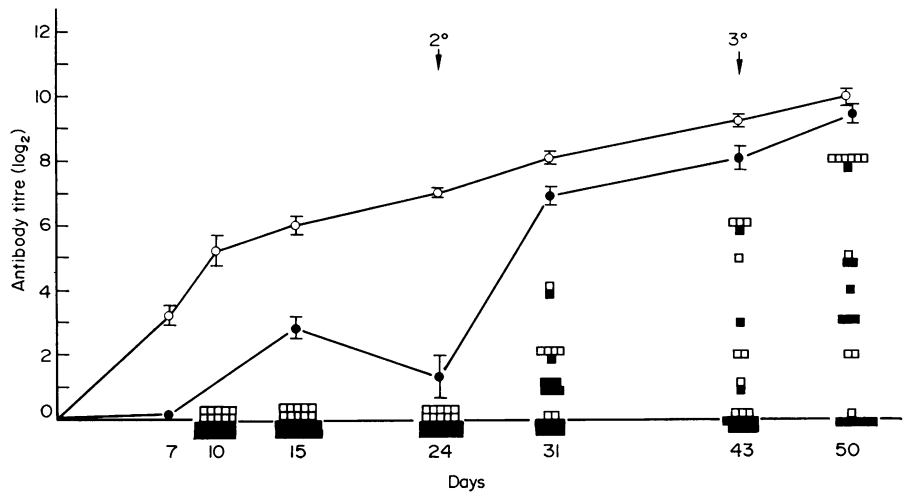

FIG. 2. Anti-SRBC (circles) and anti-HRBC (squares) titres of thymus-deprived, reconstituted mice immunized with SRBC. Open symbols are titres of control mice and closed symbols are those of mice made tolerant to SRBC.

the mean log_2 anti-HRBC titre of those that responded (4·4) was significantly less than control (6·3; $P<0·02$).

The response of the controls in these experiments confirm several points already made; particularly the absence of cross-reacting antibodies in the early primary response and the rapid rise in the secondary response.

VII. QUEST FOR ORIGINAL ANTIGENIC SIN

We performed an experiment to see whether the production of anti-HRBC antibodies in response to immunization with SRBC would alter the response to a subsequent inoculation with HRBC. Thus, we immunized one group of CBA/H mice with 5×10^8 SRBC until a significant number of them made anti-HRBC antibodies. One week after a third inoculation of SRBC 12/20 mice had anti-HRBC titres greater than 2 (mean 5·5). At this time all twenty mice, as well as seven mice which had been given SRBC, were given an inoculation of HRBC. Six days later, the anti-HRBC titres of the three groups were indistinguishable. The sera of all mice were absorbed with both SRBC and HRBC. In

Table 5 we present the results from cross-absorption of the sera of two mice given SRBC, whose anti-HRBC titres were equal 6 days after immunization with HRBC. One mouse, however, had a high anti-HRBC titres prior to immunization.

Several points can be made:

1. Dilution affected the anti-HRBC titre of the two sera differently: the presence of anti-HRBC antibodies prior to immunization with HRBC resulted in these antibodies falling less after dilution.
2. The anti-SRBC titre of both sera was abolished by absorption with SRBC but not diminished by absorption with HRBC.
3. The anti-HRBC titre of both sera was the same after three absorption with SRBC.

TABLE 5

EFFECT OF DILUTION AND CROSS-ABSORPTION ON CROSS-REACTING ANTIBODIES MADE BY MICE MULTIPLY IMMUNIZED WITH SRBC AND THEN IMMUNIZED WITH HRBC

Mouse No.	Anti-SRBC(S) and HRBC(H) antibody titres after:															
	Immunization with SRBC		Immunization with HRBC		Dilution (1:5)		Dilution (1:10)		One Absorption with				Two Absorptions with				Three Absorptions with			
									SRBC		HRBC		SRBC		HRBC		SRBC		HRBC	
	S	H	S	H	S	H	S	H	S	H	S	H	S	H	S	H	S	H	S	H
1	12	10	10	13	8	11	5	8	3	5	7	0	0	5	6	0	0	5	7	0
2	12	1	10	13	7	7	6	5	5	5	6	0	3	6	6	0	0	5	8	0

Thus, although a population of qualitatively different anti-HRBC antibodies existed in one serum (differentiated by dilution and absorption), both sera had similar titres of antibody that were not differentiable by the techniques we used. It would seem that the anti-HRBC antibody that was not absorbed by SRBC represented the response to HRBC, which was of equal magnitude in both mice.

DISCUSSION

We have presented data which show that some mice immunized with SRBC can make high titres of anti-HRBC antibodies. Special immunizing procedures were required for the production of the anti-HRBC antibodies which probably accounts for previous failures to detect them (Cunningham, 1966; Radovich and Talmage, 1968; Möller and Sjöberg 1970; Eidinger *et al.*, 1971). The conditions that are required also help shed some light on the nature of the antibodies.

Basically, these cross-reactions could result from one of two conditions. It is possible that S and HRBC share some determinants and that the cross-reactions are due to antibodies against the shared determinants. This explanation does not adequately explain all our data. The difficulty in raising the cross-reacting antibodies by immunization with either antigen, even when they are given together, suggests that the hypothetical shared antigen is not a major one on either cell. None the less the cross-reacting antibodies are very easily absorbed from the serum by either S or HRBC, with roughly equal efficiency.

Indeed, even in the occasional antiserum where the anti-HRBC titre is greater than anti-SRBC titre, absorption with either antigen removes the anti-HRBC antibodies much more efficiently than the anti-SRBC. For example, the serum from mouse No. 4 in Table 1 had a titre ($\log_2$) of 4 against SRBC and 5 against HRBC prior to absorption. After one absorption with SRBC the anti-HRBC titre fell to 0 while the anti-SRBC titre did not fall at all. Absorption with HRBC produced similar results. The serums from mice No. 5 and No. 7 in Table 1 and the pooled serums used in the studies reported in Table 3 are other examples of preferential absorption of the cross-reacting antibody from serums in which the pre-absorption anti-S and HRBC titres were not greatly disparate. Thus, the data from the absorption studies are difficult to explain if one assumes the determinant(s) with which the cross-reacting antibodies combine are infrequent on either the S or HRBC. Infrequent occurrence of the determinants is also difficult to reconcile with the excellent agglutinating power of the antisera.

Furthermore, we showed that antibodies made to a primary immunization with HRBC, which had no demonstrable affinity for SRBC, could effectively block the ability of HRBC to absorb the cross-reacting antibodies with the same efficiency as they blocked absorption of the homologous serum. This would imply that the binding sites for the two antisera were the same.

Thus in order to ascribe the cross reactions to shared determinants one must postulate the following conditions. A frequently occurring determinant(s) which is very poorly immunogenic exists on both S and HRBC. Its lack of immunogenicity cannot be caused by its being 'hidden', because of its reactions with antibody. Further, this determinant must be extremely close to the immunogenic determinants in order for the antibodies against one determinant to block absorption by the other.

Of these postulates the most conceptually difficult for us to understand is how a major cell surface antigen can be so poorly immunogenic. Even the purified capsular polysaccharide of pneumococcus III, which is itself a very poor immunogen (see Paul, Katz and Benacerraf, 1971) (indeed it induces no γG antibodies at all (Baker and Stashak, 1969)) is highly immunogenic on the pneumococcal cell membrane (Pincus, Haber, Katz and Pappenheimer, 1968) or when complexed to a carrier to which a mouse is primed (Paul, Katz and Benacerraf, 1971). (The so-called carrier effect (Mitchison, 1971)). Similarly, certain synthetic antigens may be totally non-immunogenic on their own, but potent antisera are raised against them when they are complexed to immunogenic carriers. (McDevitt and Benacerraf, 1969). There are, as far as we know, no previously reported major, non-immunogenic, cell surface determinants on immunogenic cells. Indeed, immunogenicity seems to be a property of the carrier rather than the determinant (Siskind, Paul and Benacerraf, 1966; Gershon and Paul, 1971) or as Dresser (1962) has put it, to inherent 'adjuvanticity' which should be equal for all the determinants on the SRBC.

An alternate explanation for the cross-reactions is that they result from antibodies directed to distinctive determinants on the SRBC which are able to bind distinctive but related determinants on the HRBC. This type of cross-reaction, originally analysed by Landsteiner (1947) in regard to red cell antigens, has been studied extensively by Eisen and his colleagues more recently (Little and Eisen, 1969; Little, Border and Freidin, 1969; Underdown and Eisen, 1971). They have shown that antibodies raised against a hapten, 2,4-dinitrophenyl (DNP), may bind structurally related ligands such as 2,4,6-trinitrophenyl (TNP) or even structurally unrelated ligands such as 5-acetouracil (5AU). By virtue of the fact that anti-DNP and anti-TNP antibodies differ in some fluorescence

properties, they have been able to show that the antibodies which actually bind the cross-reacting ligand retain the characteristic fluorescent properties of the general antibody population raised by immunization with the homologous (non-cross-reacting) hapten. They have further shown that the cross-reacting antibodies are those which have a particularly high binding constant (affinity) for the homologous ligand.

The cross-reactions between S and HRBC we have described are similar in many ways to those described by Eisen and his colleagues. They found the cross-reactions to occur especially late in the primary immune response or after multiple immunizations. We have also found the cross-reactions to appear at similar times. Our absorption results also suggest that of all the anti-SRBC antibodies present, the subset that also binds HRBC is the most avid for the SRBC, as it is the most easily removed by absorption. The equal absorption by both S and HRBC need not imply equal affinity or avidity of the antibody for the two cells. Using HRBC for absorption would reduce the competition for sites by the lower affinity antibodies as well as by those antibodies of high affinity which do not cross-react with the determinants on the HRBC but which might cross-react with other ligands (Underdown and Eisen, 1971). Thus the HRBC effectively may contain considerably more absorption sites per cell. Another observation which causes us to think the cross-reacting antibodies have a higher than average affinity for SRBC is one we made during our studies on the cross-blocking effects of different antisera. We absorbed the cross-reacting antibody with SRBC and the titre ($\log_2$) fell from 9,9 (vs. S & H respectively) to 9(S), 3(H). We then recovered the cells used for absorption and incubated them in normal mouse serum. A significant amount of antibody eluted from the cells giving the normal serum a titre of 8(S), 1(H). The eluate thus was particularly deficient in antibodies which could agglutinate HRBC, indicating that the cross-reacting antibodies were not only preferentially absorbed from the serum by SRBC, but once absorbed they were also preferentially bound.

The selective absence of the cross-reacting antibodies in the sera of mice which have recently escaped from tolerance is further evidence of their association with high affinity molecules (Theis and Siskind, 1968; Brown and Glynn, 1969; Bast, Manseau and Dvorak, 1971). Further, along these lines, we have previously shown that absorption of these antibodies with HRBC, from a population of anti-SRBC molecules, significantly alters the feedback effects of the antibody (Gershon and Kondo, 1971b). Such an effect is hardly likely to be produced by the removal of antibody against determinants to which mice are not responding. One further similarity between our results and those of Eisen's group is that in both cases the cross-reacting antibodies, although highly avid, represent a minor subset. We were able to absorb them entirely without reducing the anti-SRBC titre.

Thus, on the basis of the close relationship between our results and those of Eisen and his colleagues as well as the difficulty in explaining our results with alternate hypotheses, we favour the interpretation that the cross-reactions we have observed are due to the reaction of high affinity molecules with related ligands. Should this interpretation prove to be incorrect it would still seem that the cross-reactions may be useful markers for the presence of high affinity molecules as we have noted their presence only under conditions where we would expect to find antibodies with relatively high binding constants (Eisen and Siskind, 1964; Nussenzweig and Benacerraf, 1967; Steiner and Eisen, 1967; Goidl *et al.*, 1968).

It should be emphasized that the presence of high affinity or avidity antibodies, as signalled by these cross-reactions, does not necessarily mean that the antiserum which

contains them will have a high *average* affinity. For example, the presence of large numbers of low affinity antibodies *might not reduce* the cross-reactions but would affect values for average affinity as determined by equilibrium dialysis. We suspect that some of the sera from hyperimmunized mice also contained large amounts of low affinity molecules because much more SRBC was needed to absorb them to zero titre than was necessary for equally titred sera from mice immunized less often. Thus, this technique of affinity determination may detect high affinity antibodies in highly heterogeneous antisera where other methods, which measure average affinity, would fail to detect them in the presence of much low affinity antibody.

An important question in light of these cross-reactions is whether the use of SRBC and HRBC as antigen pairs in experiments on antigenic competition require re-evaluation (Radovich and Talmage, 1967; Albright, Omer and Dietchman, 1970; Möller and Sjöberg, 1970; Eidinger *et al.*, 1971; Gershon and Kondo, 1971a, b; O'Toole and Davies, 1971). We believe the interpretation that the effect is non-specific rather than due to cross-reactions is still correct; particularly in view of the demonstration that KLH or PVP can be used as substitutes for one of the red cells (Eidinger *et al.*, 1971). Furthermore, Miller and Mitchell (1968, 1970) have shown that the production of tolerance to SRBC with cyclophosphamide does not affect the immune response to HRBC and we recently showed the same thing with a different method of tolerance induction (Gershon and Kondo, 1971c). Mitchell and Miller (1968) have also shown that immunization of thymocytes with HRBC does not increase their ability to reconstitute the anti-SRBC response of thymus-deprived mice. Also, we have reported that the passive transfer of cross-reacting antibodies produced by immunization with SRBC does not diminish the immune response of mice to HRBC while it abolishes their response to SRBC (Gershon and Kondo, 1971b). These results all suggest a distinctive mechanism for their production.

Our experiments on original antigenic sin (Table 5) support this conclusion. We showed that, mice which made anti-HRBC antibodies as a result of immunization with SRBC, as well as those that failed to do so, both made equal amounts of non-cross-reacting anti-HRBC antibody after HRBC immunization. Cross-reactions based on related ligands might be expected to exhibit such distinctions as Little and Eisen (1969) have shown that the precursor cell populations stimulated by each ligand are virtually non-overlapping.

Our report of degeneracy of the immune response to cellular antigens, adds emphasis to and perhaps broadens the implications of the previous reports of this phenomenon. Thus, the use of hyperimmunization to obtain antisera may yield confusing cross-reactions. A possible example of this can be found in the recent controversy (see *Science* (1971), (*Race for Human Cancer Virus: Odds against Houston team lengthen*, **73**, 1220) as to whether the Type C virus isolated from the ESP-1 cell line (Priori, Dmochowski, Myers and Wilbur, 1971) is of mouse or human origin. Although several laboratories have evidence that the virus is distinct from mouse viruses, a recent report has shown that immunodiffusion tests with sera specific for the mouse virus group-specific antigen show a precipitin line of identity between murine group-specific antigen and concentrates containing the virus liberated by the human cells (Gilden, Parks, Huebner and Todaro, 1971). This has led to a leader in *Nature* (1971, **88**, 233) stating that the antigen is most likely a contaminant and a red herring. No information is given anywhere as to how the antisera were raised nor were any cross-absorption studies done. Little and Eisen (1969) have shown that reactions of identity will occur in immunodiffusion tests with anti-DNP antibodies tested against DNP and TNP determinants unless suitably selected anti-DNP subsets are iso-

lated. Thus, reactions of identity do not necessarily mean identical antigens particularly if the reference antiserum is one with significant amounts of high affinity antibodies. This is an extremely important point and is often ignored. Other recent examples where these considerations may significantly alter the interpretation of data may be cited (Bonavida and Sercarz, 1971).

In summary, we have shown that a striking parallel exists between the degeneracy of the immune response to SRBC and that to other better defined antigens. We have discussed why we think the basis for the phenomenon is similar in the two cases. However, we wish to emphasize that, independent of the underlying mechanism, these observations broaden the generalization that the immune response loses specificity as it progresses. This observation has important theoretical and practical implications.

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