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Relationship between Skin Transplantation Immunity and the Formation of Humoral Isoantibodies in Mice

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Summary. Crude splenic nuclear fractions can incite skin transplantation immunity in mice without producing a detectable titre of haemagglutinins or haemolysins, and have little if any power to absorb antibodies from sera in which they have been caused to appear by other means. Antigenic preparations of other kinds can incite iso-antibody formation unaccompanied by transplantation immunity. Thus humoral iso-antibodies can be present when transplantation immunity is absent and absent when it is present. Transplantation immunity and serum antibody production probably depend upon different immunological processes, even if they eventually prove to be elicited by antigens with the same or similar determinant groups.

INTRODUCTION

IT has been known for twenty years that the rejection of tumour homografts by mice is accompanied by the formation of serum antibodies (Gorer, 1937, 1938), and the same is now known to be true of homografts of skin (Amos, Gorer, Mikulska, Billingham and Sparrow, 1954). The antibodies so formed are usually titrated as haemagglutinins, but they can also be titrated as cytotoxins (Gorer and O'Gorman, 1956), haemolysins (Hildemann, 1957) or leuko-agglutinins (Amos, 1953). It cannot be assumed that these several test systems simply reveal different expressions of the action of a single type of antibody. On the contrary, there is good evidence that the cellular iso-antigens provoke the formation of antibodies of several different kinds (Gorer, 1956; Amos and Day, 1957; Feldman and Sachs, 1957).

The part played by cell-free antibodies in the homograft reaction is still in question, for there is evidence which suggests that the reaction which causes the destruction of 'solid' tissue homografts—of homografts which establish primary vascular connections with their hosts—is cellular rather than humoral, having more in common with the tuberculin reaction and with hypersensitivities of the delayed type, than with those immunological reactions in which humoral antibodies play some necessary part (Mitchison, 1954; Medawar, 1954; Mitchison and Dube, 1955; Brent, Brown, and Medawar, 1958; Berrian and Brent, 1958).

Experiments on both mice (reviews by Kaliss, 1955, 1957) and goldfish (Hildemann, 1958) have made it clear that haemagglutinins or other humoral antibodies may be present when transplantation immunity is absent. (In mice, indeed, the work of Kaliss and his colleagues has shown that the presence of a high titre of circulating antibodies is causally connected with a weakening of transplantation immunity, i.e. 'enhancement'.) Evidently the formation of haemagglutinins cannot be sufficient to account for transplantation immunity. If the converse could also be shown to be true, viz. that transplantation immunity can be in force when haemagglutinins and haemolysins are absent, then

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one could draw the stronger inference that antibodies of this kind play no necessary part in the homograft reaction. This is the problem we report upon in the present paper. Our experiments consist essentially in showing that antigens known to be capable of producing transplantation immunity can do so without producing a detectable titre of haemagglutinins or haemolysins, and have little if any power to absorb haemagglutinins and haemolysins from sera in which they have been caused to appear by other, efficacious, means.

The nature of the antigens that cause the various iso-immune reactions accompanying the rejection of homografts is still obscure, for it was only two years ago that antigens capable of causing transplantation immunity were shown to be present in disintegrated cells and in extracts made from them (Billingham, Brent and Medawar, 1956a). 'Transplantation antigens' are known to be present in nuclear fractions of splenic and other lymphoid cells. It was originally believed that they were either deoxyribonucleoproteins or substances integrally associated with deoxyribonucleoproteins, but more recent analyses almost certainly exclude the direct participation of deoxyribonucleic acid and point rather towards a determinant group of mucoid nature (Billingham, Brent and Medawar, 1958). Kandutsch and Reinert-Wenck (1957) have shown that iso-antigens capable of enhancing the growth of tumour homografts occur in both nuclear and cytoplasmic fractions; their activity appears to depend upon the presence of both carbohydrate and protein, possibly in the form of a mucoprotein. They found, moreover, that hexosamines may be present in even highly purified deoxyribonucleoproteins. In the experiments described here we have mainly used washed splenic nuclei or nuclear fragments as a source of 'transplantation antigens', but the significance of the experiments does not turn upon any particular interpretation of their chemical nature.

EXPERIMENTAL METHODS

The subjects of these experiments were mature mice of domestic sublines of strains A and CBA. In respect of the important antigens determined by the H-2 locus (see Gorer, 1956) the antigenic composition of A-line mice is CDEFK and of CBA mice is CdEfK. The combination in which A-line mice are donors and CBA mice recipients is particularly favourable for serological analysis, and was chosen accordingly. The reciprocal combination, in which CBA cells or antigens are used to immunize A-line mice, is much less satisfactory (Table 1, exp. 11). This difference does not reveal itself in the survival times of skin homografts, which are much the same either way: A→ CBA, 11·0±0·3 days; CBA→ A, 10·2 ± 0·3 days (Billingham, Brent, Medawar and Sparrow, 1954).

SEROLOGICAL METHODS

Most antisera were titrated in parallel by three methods: (*a*) by haemagglutination in the presence of dextran and absorbed human serum—essentially the technique of Gorer and Mikulska (1954); (*b*) by haemagglutination, as above, but substituting isologous (A-line) serum for human serum; and (*c*) by haemolysis in the presence of rabbit complement, the technique of Hildemann (1957).

The agglutination tests (*a*, *b*) were done in polished plastic trays each containing 80 hemispherical cavities of 1·5 cm. diameter. Reagents were mixed in the ratio 0·1 ml. antiserum in doubling dilutions in 2 per cent dextran to 0·1 ml. of a 2 per cent suspension

TABLE I

ANTIBODY TITRES SECURED BY THE INJECTION OF A-LINE CELLS OR CELL-FREE ANTIGENS INTO CBA MICE

Exp. No.	*No. of recipients*	*Dosage of cells or cell preparation per injection per mouse (intraperitoneal)*	*Days of injection*	*Serum taken on days after last injection*	*Reciprocal of red-cell antibody titres found in three systems**		
					Dextran-haemagglutination		*Haemolysis, rabbit complement*
					isologous mouse serum	*absorbed human serum*	
1	2	20 mg. lyophilized kidney	0, 6, 9, 13, 29, 32	10	32, 32	—	—
2	3	75·5 mg. wet weight spleen (=166 × 10^6 non-red cells)	0, 6, 9, 13, 29, 32	10 10	512, 1024, 1024 4096, 4096, 8192	— 2048, 4096	— 1024
3	3	Nuclear fragments from 150 mg. wet weight of spleen	0, 3, 7, 10	11	0, 0, 0	8, 16	0, 0, 0
4	4†	20 mg. lyophilized kidney	0, 5, 8, 12, 15 37	10 10	16, 16 16, 64	— 64, 128	— 0, 0
5	3	Nuclear fragments from 180 mg. wet weight of spleen	0, 3, 7, 10	10	0, 0, 0	—	—
6	4	58 mg. wet weight spleen (=128 × 10^6 non-red cells)	0, 3, 6, 10	12	256, 512, 512, 1024	512, 1024, 1024, 2048	256, 256, 512 512
7	3	129 × 10^6 splenic nuclei plus ~0·529 × 10^6 intact cells	0, 3, 6, 10	12	32, 32, 64	256	4
8	3	50 mg. wet weight spleen (=110 × 10^6 non-red cells)	0, 4, 10, 12 28	11 9	512, 1024, 1024 —	512, 2048, 2048 2048‡	128, 512, 1024 1024‡
9	3	Nuclear fragments from 500 mg. wet weight of spleen	0	10	0, 0, 0	0, 0, 0	0, 0, 0
10	3	Nuclear fragments from 250 mg. wet weight spleen	0, 3, 7, 11, 22	7 10	256 256, 256	256, 256, 256 256, 256, 512	32, 32, 64 16, 16, 16
11§	3	100 mg. wet weight CBA spleen (=221 × 10^6 non-red cells)	0, 2, 5, 8, 21	10 7	— —	— 0, 0, 0	0, 0, 0 0, 0, 0
12	3	560,000 splenic cells	0, 3, 7, 11, 22	7 10	— —	512, 512, 1024 512, 512, 512	512, 1024, 1024 512, 512, 1024

* Because of the limited quantities of serum available for testing, the number of titres given for each system does not always correspond to the number of recipients.
† Two animals died of anaphylactic shock after the last injection.
‡ Pooled serum.
§ CBA donors and A-line recipients.

of A-line erythrocytes in *either* 50 per cent v/v absorbed human serum (method *a*) *or* 50 per cent v/v normal A-line serum (method *b*). The agglutination trays, covered with a glass plate sealed on with silicone grease, were incubated for 2 hr. at 37°. The degree of agglutination was ascertained by reflected light under a binocular microscope, but samples were streaked upon slides to verify end-points. In control tests, the antiserum was replaced by 2 per cent dextran. Glaxo 'Intradex' 10 per cent dextran (salt-free) in 5 per cent glucose, and 0·85 per cent NaCl with or without phosphate buffer at *p*H 7·4, were used for the required dilutions.

Haemolytic tests (*c*) were performed in miniature test tubes, the reagents being mixed in the following proportions and order: 0·2 ml. antiserum in doubling dilutions, 0·1 ml. 2 per cent A-line erythrocytes, and 0·1 ml. 20 per cent v/v rabbit complement. After 90 min. at room temperature the tubes were spun for 40 sec. at about 600**g** and the supernatants inspected for haemolysis. Final readings to verify end-points were taken after an additional hour at room temperature. All dilutions were made with 0·85 per cent phosphate-buffered saline containing 0·01 per cent $MgCl_2.6H_2O$.

PREPARATION OF ANTIGENS

Except in Exp. 11, Table 1, all the agents used for injection into mice or for the absorption of antisera were of A-line origin. They comprised 'lyophilized' kidney; living splenic cells; splenic nuclei or nuclear fragments; 'soluble antigen' from spleens; washed erythrocytes. All immunizing injections were intraperitoneal.

Lyophilized kidneys were prepared in bulk by orthodox methods and stored at 4° over P_2O_5 awaiting use. Suspensions of splenic cells were made by pressing coarsely chopped spleens into Ringer-phosphate through a stainless steel sieve. The cell counts in Table 1 refer to nucleated cells: A-line spleens weigh 80 to 100 mg. and yield about 220×10^6 nucleated cells per 100 mg. wet weight. Splenic nuclei were prepared by washing such a suspension two or three times in Ringer-phosphate and then exposing it for 60 sec., in successive 5 to 7 ml. volumes, to ultrasonic vibrations produced by an MSE-Mullard 20 kc/s 50W generator of the magnetostrictive type. (In this apparatus the sound waves are transmitted to the surface of the solution under treatment through a half wave length stainless steel stub coupled to the transducer.) After exposure the preparation, containing whole and broken nuclei, was washed three times in a large excess of Ringer-phosphate. Nuclear fragments were prepared in the same way except for the substitution of normal (0·15M) NaCl solution for Ringer-phosphate at all stages: in the absence of divalent cations all the nuclei are disintegrated into small fibrous fragments. Intact cells in measurable numbers were still present in the nuclear preparation (Exp. 7, Table 1); they were certainly much less frequent among the nuclear fragments prepared in plain saline solution, but cannot be assumed to have been absent.

'Soluble antigen' from A-line spleens was made by a rather unsatisfactory method which has since been superseded (Billingham, Brent and Medawar, 1958). Cells expressed from A-line spleens into 0·15M NaCl were washed successively, by centrifugation, in 0·15M NaCl, 0·05M NaCl and water. The gelatinous sediment left after the last centrifugation was smoothed out in a blender of the piston and cylinder type, exposed to ultrasound as above, and spun at 2000**g** for 10 min. to remove undispersed matter. Magnesium chloride solution was added to the supernatant to bring its final concentration to 0·01M and the clear white precipitate, containing almost all the antigenic matter of the

TABLE 2

ABSORPTION OF ANTISERA WITH VARIOUS A-LINE ANTIGENIC PREPARATIONS

Exp. No.	*Preparation used in absorption*	*Absorption procedure*	*Quantity of antiserum mixed with preparation for absorption*	*No. of tests*	*Dextran haemagglutination titre*	
					absorbed serum	*unabsorbed serum**
13	Washed A-line nuclear fragments from 850 mg. wet weight of spleen	0·12 ml. packed fragments in each of 3 tubes: each absorption 30 min. at 20°	0·5 ml. at 1:16 in 2% dextran	2	64, 64	128, 128
14	Washed A-line nuclear fragments from 940 mg. wet weight of spleen	0·14 ml. packed fragments in each of 3 tubes. First absorption, 30 min. 37°; last two 30 min. each, 20°	0·5 ml. at 1:32 in 2% dextran	2	2048, 2048	8192
15	Washed A-line red cells	0·15 ml. packed red cells in each of 3 tubes: absorptions as in exp. 14	0·5 ml. at 1:32 in 2% dextran	2	no reaction	8192
16	A-line 'soluble antigen' from 1·23 g. wet weight of spleen	1·55 ml. 'soluble antigen' in one tube; absorption 90 min., 20°	0·05 ml. to give 1:32 dilution in 2% dextran	2	2048, 2048	8192
17	A-line 'soluble antigen' from 1·00 g. wet weight of spleen	6·35 ml. 'soluble antigen' in one tube; absorption 90 min. 21°	0·05 ml. to give 1:128 dilution in 2% dextran	2	1024, 1024	4096
18	Washed A-line nuclear fragments from 1·04 g. wet weight of spleen	0·20 ml. packed fragments in each of 3 tubes. First two absorptions, 30 min. 20°; last, 30 min. 37°	0·25 ml. at 1:32 in 2% dextran	1	no reaction†	2048
19	Washed A-line nuclear fragments from 1·61 g. wet weight of spleen	0·25 ml. packed fragments in each of 3 tubes: absorptions as in exp. 18	0·40 ml. at 1:32 in 2% dextran	2	256, 256	2048, 2048

* *Sources of immune sera:* exp. 13, see exp. 2, Table 1 (initial serum); exps. 14 to 17, see exp. 2, Table 1 (hyperimmune serum); exps. 18, 19, see exp. 8, Table 1 (pooled antiserum).

† Accidental failure to suspend nuclear fragments in 2% dextran led to excessive dilution of antiserum-dextran in absorptions.

parent solution, was thrown down by centrifugation. It was thereupon digested for 15 min. at 37° by crystalline trypsin (1 mg./ml. in Ringer-bicarbonate) to render it soluble in salt solution of 0·15M molarity. An empirically determined equivalent of purified soya-bean trypsin inhibitor was added to the digest, and undissolved matter removed by centrifugation. The final preparation was diluted to 2 per cent with respect to dextran and used in absorption tests with antiserum to A-line cells.

Antigenic preparations were tested for their power to incite transplantation immunity by the method of Billingham, Brent and Medawar (1956a). A single injection of the nuclear fragments or soluble antigen extracted from 200–250 mg. wet weight of spleen is capable of eliciting the maximal response that can be recorded by this method, viz. the total breakdown, within six days, of a skin homograft transplanted three or four days after the immunizing injection.

Living cells and antigens extracted from them were injected according to a time-schedule suitable for the production of antibodies in high titre, a fact which gives added significance to the absence of antibodies in the experiments in which they were not found.

RESULTS

The results are summarized by Tables 1 and 2. In Table 1, Exps. 2, 6 and 8 show that high titres of agglutinins and lysins are produced by repeated injections of living splenic cells in numbers of the order of 10^8. The differences in serum titres of individual mice within any one experiment rarely exceeded one doubling dilution more or less. Exp. 9 shows that a single massive injection of nuclear fragments—at least twice as great as that necessary to produce the maximal response in the test for transplantation immunity—was unable to elicit a detectable titre of antibodies. The same is true of repeated injections of smaller quantities (Exps. 3, 5). Repeated injections of higher doses of nuclear fragments according to a time schedule which allowed for a rest followed by a 'boosting' dose yielded (Exp. 10) moderate titres of agglutinins and rather low titres of lysins. The 250 mg. wet weight of splenic tissue used in the preparation of the nuclear fragments for each injection in Exp. 10 would have yielded, on the average, 55×10^7 nucleated cells. Exp. 12 shows that the antibodies formed in Exp. 10 could be fully accounted for if one cell in a thousand had escaped destruction during the preparation of nuclear fragments, for in Exp. 12 even higher titres of antibodies were produced when as few as 55×10^4 living splenic cells were injected according to the time schedule adopted in Exp. 10. Avowed contamination with whole cells could also account for the antibodies formed in low titre after repeated injections of splenic nuclei (Exp. 7).

The injection of living CBA splenic cells into A-line mice (Exp. 11) in doses even higher than those used in the reciprocal combination (Exps. 2, 6, 8) produced no detectable serum antibodies.

The consequences of injecting lyophilized kidney tissue (Exps. 1, 4) are particularly interesting, because injections of this kind are known to 'enhance' rather than discourage the growth of tumour homografts (see above) and to prolong the normal lifetime of homografts of skin (Billingham, Brent and Medawar, 1956b). The injections led to the formation of a low but far from negligible titre of agglutinins, but haemolysins could not be detected.

The absorption experiments are summarized in Table 2. Exp. 15 confirms the adequacy of the technique of absorption by the use of A-line red cells. Three successive absorptions with washed, packed nuclear fibres extracted from up to one gram wet weight of spleen

(Exps. 13, 14, 16) produced no greater diminution of titre than dilution of antibodies might reasonably account for. The apparently complete absorption recorded from a similar preparation in Exp. 18 was almost certainly due to the technical error referred to in the table. The reduction of titre produced by the relative high bulk of absorbing fibres in Exp. 19 is probably insignificant, because the loss of approximately two doubling dilutions might be accounted for by dilution alone during the three absorptions. However, no control experiment was done to assess the magnitude of non-specific absorption or the degree of contamination by whole cells. The experiments (16, 17) in which 'soluble antigen' in relatively very high concentrations was used to absorb antibodies from immune serum may be thought particularly indicative: here the titre of immune serum was reduced by only two doubling dilutions.

DISCUSSION

The experiments described above show that antigens which are capable of eliciting transplantation immunity may do so without producing any detectable titre of iso-agglutinins or isolysins, in spite of the fact that both the schedule of injections and the strain combination (A→ CBA) were chosen to favour the formation and detection of humoral antibodies. There is no clear evidence that the crude nucleoprotein-containing fraction used to elicit transplantation immunity can either produce free serum antibodies or absorb them from sera in which they have been produced by other means.

One possible explanation of these results is that the difference between serum antibody formation and transplantation immunity is merely one of degree. If the 'homograft test' for transplantation immunity were very much more sensitive than the serological test for antibodies, then doses of antigen which were quite adequate to bring about the accelerated breakdown of a test homograft might well fall far short of what would be necessary to produce a serologically detectable titre of antibodies. But if this were so, then even the low titre of serum antibodies produced by the injection of lyophilized kidney tissue (Table 1, Exps. 1, 4) should have been associated with a transplantation immunity still more violent than that which can be in force when no antibodies can be detected at all. The injection of lyophilized tissues tends, however, to weaken resistance to skin homografts (see above), and reference has already been made to the fact that the intraperitoneal injection of homologous whole blood into goldfish can produce haemagglutinins without transplantation immunity (Hildemann, 1958). Thus it is indeed possible to have transplantation immunity in the absence of detectable haemagglutinins and haemolysins, and, conversely, to have no transplantation immunity in their presence.

It is difficult to avoid the inference that transplantation immunity and serum antibody formation represent two different kinds of immunological response. Other evidence that the homograft reaction is essentially 'cellular' in its manner of execution, or at least not humoral, is as follows: (*a*) transplantation immunity can be passively ('adoptively') transferred by activated lymphoid cells, but not by serum (Mitchison, 1954; Billingham, Brent and Medawar, 1954; Billingham and Brent, 1956), and can manifest itself in the guinea pig as a hypersensitivity reaction, apparently of the 'delayed' type, which is likewise transferable by sensitized lymphoid cells but not by immune serum (Brent, Brown and Medawar, 1958). Then (*b*) homografts survive within highly sensitized hosts provided that they are protected by membranes which, though permeable to blood solutes, will not allow cells to pass through (Weaver, Algire and Prehn, 1955; Algire, 1957); (*c*) the times

of inception of transplantation immunity and humoral antibody formation are clearly different: the former is the quicker to arise (Mitchison and Dube, 1955); and (*d*) foetal lambs and newborn calves are capable of reacting against homografts before they are known to be capable of synthesizing gamma globulins (Schinkel and Ferguson, 1953; Billingham and Lampkin, 1957).

Are the two different immune responses caused by the same or by different antigens? No definite answer can yet be given. In favour of their being the same, or closely similar, are (*a*) the fact that some, but by no means all of the antigens causing enhancement, transplantation immunity, and the formation of humoral antibodies have a common genetic determination at the H-2 locus; and (*b*) the possibility (referred to in the *Introduction*) that the determinant groups of the antigens causing enhancement and transplantation immunity may be of a mucoid nature. The demonstration by Kandutsch and Reinert-Wenck (1957), that even quite highly purified deoxyribonucleoproteins still contain hexosamines and so, by inference, mucoid substances, may be thought sufficient to account for the presence of 'transplantation antigens' in our washed nuclear fragments. Snell (1957) has accordingly suggested either that deoxyribonucleoprotein acts as an adjuvant which favours the cellular rather than the humoral pathway of response, or—following Kandutsch—that a mucoid antigen might be present in nuclear preparations 'in unstable coupling with some other substance, perhaps a lipid, which altered the type, but not necessarily the specificity of the recipient's response'. Nevertheless the failure of splenic nuclear fragments or 'soluble antigen' to absorb the antibodies evoked by living cells of the same origin, and the inability of nuclear preparations to induce the formation of such antibodies, argue in favour of the view that different antigenic specificities underlie the two responses. It is significant that red cells, which in our experience do not produce skin transplantation immunity, are quite capable of absorbing serum antibodies elicited by splenic cells.

Transplantation immunity and the production of humoral antibodies may represent two different pathways of response that diverge from the outset, or, alternatively, the cellular activation associated with the homograft reaction may represent an episode in a process which ultimately leads to the formation of humoral antibodies. One difficulty in the way of accepting this second hypothesis is the lack of any clear evidence that the incitement of transplantation immunity is a necessary first step towards procuring 'enhancement'. On the contrary, mice which have been sensitized with agents that produce transplantation immunity become for some time refractory to treatment with the lyophilized tissues, tissue extracts or antisera that are used to enhance the growth of homografts.

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