Transplantation antigens *per se* are poor immunogens within a species

THE structure of HLA substances make it surprising that they provoke strong alloimmune responses[1-3]. They are composed of two chains, one invariant within the species, and the other bearing a single determinant defined by monospecific HLA sera[4], or a few determinants defined by polyspecific sera[4,5], and thus most closely resemble haptens on non-immunogenic carriers. If this is true generally of antigens determined by major histocompatibility systems (MHC), they could be expected to be poorly or non-immunogenic within a species. This, however, conflicts with the evidence that when present on viable tissue grafts, these antigens provoke strong humoral and cellular alloimmune responses[6-8]. But there is also evidence that their immunogenicity depends on additional factors[9,10]. Here, we present data which reconcile these seemingly conflicting lines of evidence, by showing that the induction of primary immune responses (humoral and cellular) against MHC antigens *in vivo* depends both on the presentation of alloantigen and on a second signal provided by a live cell. Without the latter, the small quantities of MHC antigen present on conventional allografts would not induce primary immunity.

The two sets of observations which showed that MHC antigens are poorly or non-immunogenic within the species are that mouse red cells, which are known to carry H-2 antigens, fail to induce second set skin allograft rejection[9], and that allogenic rat platelets fail to induce primary humoral or cellular immunity against Ag-B determinants[10]. Our calculations indicated that 10^{-9} g of Ag-B glycoprotein when present on viable lymphocytes could induce a primary cytotoxic antibody response (titre 1/100–1/128), but that 10^{-5} g of Ag-B antigen when present on platelets failed to do so. As the primary response to MHC antigens is T-cell dependent[11], we suggested that Ag-B antigen by itself is insufficient to activate helper T cells. Because of the evidence implicating I-region determinants in the activation of helper T cells[12,13], the induction of a primary response by lymphocytes, but not by platelets was attributed to the presence of Ag-B and Ia-like determinants on the same cells. In order to analyse further the question of what makes Ag-B immunogenic, we have measured immune responses in rats injected repeatedly with 3.5×10^{-7} g of Ag-B antigen (assuming 10^5 antigen molecules per lymphoid cell[14]) bound to carriers, bearing or lacking Ia-like determinants.

Figure 1*a* and *b* illustrates the antibody responses of AS (Ag-B^1) rats injected with membranes prepared from August strain (Ag-B^5) spleen and lymph node cells or from liver[15], liposomes containing Ag-B^5 alloantigen, or intact, viable, but heavily-irradiated (2,500 R) August spleen and lymph node cells. All inocula were adjusted to contain equal amounts of Ag-B determinants, based on the results of quantitative absorptions. All inocula except the liver membranes were also demonstrated by quantitative absorptions to have approximately equal amounts of rat Ia-like antigen. In contrast, the liver membranes contained Ag-B but no detectable Ia-like antigens. A striking difference in immunogenicity was observed, depending on whether the MHC antigens were presented on viable, but non-proliferating, lymphoid cells, or on membranes or artificial membranes (liposomes). Only intact spleen and lymph node cells, with or without complete Freund's adjuvant stimulated cytotoxic antibody responses in the unprimed rats; in the primed rats both Ag-B- and Ia-like antibodies appeared in recipients challenged with spleen and lymph node cell membranes, liposomes, or intact lymphoid cells. Liver membranes induced only Ag-B antibody in the primed recipients. In other experiments (not shown) tumour cell membranes bearing both Ia and Ag-B antigens behaved in an identical manner to spleen and lymph node membranes. Figure 1 also shows that repeated

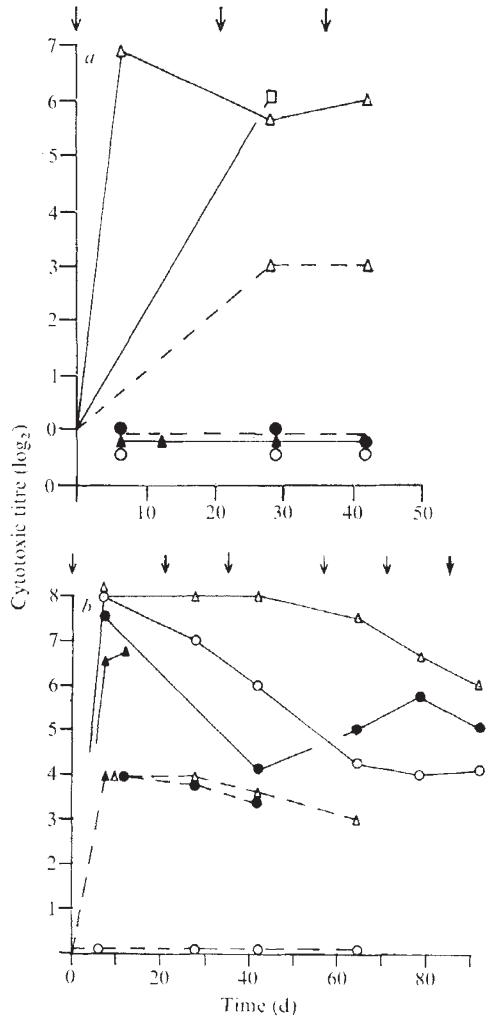

Fig. 1*a*, AS strain rats were injected with August strain liver membranes (○), spleen and lymph node membranes (●), liposomes bearing August strain Ag-B and Ia-like determinants (Willoughby, E., Turner, M. J. & Sanderson, A. R., in preparation) (▲), irradiated spleen and lymph node cells (2,500R) with (□), or without (△) complete Freund's adjuvant on the days indicated by the arrows. Each injection was equivalent to 3.5×10^7 August lymphoid cells in terms of Ag-B content. Animals (four per group) were tested for cytotoxic antibody titres, Ag-B response (———) being monitored against August lymph node cells and the Ia response (– – –) being monitored against Wistar B cells (August and Wistar share Ia but not Ag-B)[10]. Titres are expressed as means of each group (end point = 50% cells killed); animals in each group were either all positive or all negative. s.d., all less than 1, except for two points in *b* where for rats injected with spleen and lymph node membranes, the Ag-B mean titres on days 42 and 65 had s.d. of 1.1 and 1.25 respectively. *b*, As above, but rats were primed several months before these experiments and immune response was allowed to decrease to non-detectable levels before the boosts.

injections of membranes or liposomes into non-primed recipients did not succeed in exciting an antibody response; similarly (data not shown), the injection of spleen and lymph node membranes or liver membranes emulsified in complete Freund's adjuvant also failed to elicit antibody production in unprimed rats. In further experiments (not shown), DNP- or BSA- conjugated allogeneic platelets did not induce primary alloantibody responses, demonstrating that the lack of response to Ag-B was not due to the absence of a second epitope which provides essential 'help'.

Cell-mediated immunity (CMI) was measured by ^{51}Cr release from August target cells[16] incubated for 4 h with peritoneal effector lymphocytes from injected rats. August liver membranes (Table 1), and spleen and lymph node membranes or liposomes (data not shown) provoked only feeble cellular immunity in both primed and unprimed rats.

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Table 1 CMI response of AS rats given August liver cell membranes

Group	Effector–target cell ratio	Day 7	11	18	28	42
Primed	100:1	4.82†	3.63	4.63†	8.57†	0.76
	50:1	3.03*	2.89	2.65	6.00†	0.52
	25:1	2.37	2.56	0.96	3.96*	0.50
Unprimed	100:1	6.77†	4.00†	2.04	10.67†	4.44
	50:1	ND	ND	0.54	6.78*	ND
	25:1	ND	ND	ND	2.69	ND
Hyperimmune	100:1	44.25†	49.26†	57.26†	62.38†	58.98†
	50:1	42.94†	45.86†	48.45†	61.23†	47.75†
	25:1	38.99†	41.82†	44.03†	56.79†	40.46†

Primed AS rats were given 2×10^7 August lymphoid cells, i.p. on day −120. Primed and unprimed AS rats were given August liver cell membranes approximately equal to 3.5×10^7 lymphoid cells, i.v. on days 0, 21 and 36. Peritoneal exudate lymphocytes taken from injected rats, on the days indicated, were tested for lysis of ^{51}Cr-August thymus cell targets. Spontaneous release (already subtracted) varied between 6 and 14% of the total count. Results are expressed as means of triplicates, those significantly above the spontaneous release level are indicated.
*$P < 0.05$; †$P < 0.01$.

Because isotope release was so low in the case of cultures containing effector cells from membrane-treated rats, no attempt was made to investigate specificity of the response at that stage. The rats were further immunised at the intervals indicated and retested for specific immunity on days 64 and 92 (Table 2). The additional injections of either liver or spleen and lymph node membranes did not lead to any substantial increase in cellular immunity. At day 92, after five spaced, boosting injections, even primed rats injected with membranes showed less than 6% specific ^{51}Cr release at an effector–target cell ratio of 100:1. This contrasts with the development of strong and specific cellular immunity in primed rats injected with intact lymphoid cells.

It is clear from these and earlier results that MHC epitopes *per se* do not have unusually powerful alloimmunogenic properties; this generalisation applies to both the classical Ag-B determinants, and the rat equivalent of Ia antigens. Our data show that to be strongly immunogenic, the antigens must be presented on viable cells, but not necessarily replicating ones, as heavily irradiated cells provide an adequate stimulus. The experiments do not show whether Ag-B or Ia alone can immunise if on a live cell, and to examine this point we have injected allogeneic cells which either lack or have low Ia antigen content (Table 3). The results suggest that the responses to H-2 or Ag-B on viable cells does not correlate with the concentration of Ia antigen present.

The interesting point about MHC antigens is that they are strongly alloimmunogenic when present at ng levels but only when present on viable cells. In contrast, immunoglobulin allotypes which also approximate to haptens on non-immunogenic carriers require μg to mg amounts of immunogen with adjuvant in order to induce allotype antibodies. The immunogenic potency of HLA and other MHC components is also entirely different across a species barrier, but in such cases, the number of 'foreign' epitopes is such that the molecule no longer approximates to a hapten on a non-immunogenic carrier. The requirements for Ag-B to induce a primary response *in vivo*, that is, the presence of an intact nucleated stimulating cell, not necessarily capable of division, are suspiciously like those which have to be fulfilled for the generation of blasts or cytotoxic-T cells *in vitro*[12,17]. Lafferty and Woolnough[17] have proposed a two-signal activation hypothesis for immune responses to H-2 antigens. Our data fit this idea and also show that two signals are needed for activation of res-

Table 2 CMI response of AS rats given August cell membranes

Group	Injection	Effector–target cell ratio	Day 64 Net. rel. *v.* August	Day 64 Net. rel. *v.* AS	Day 64 Spec. rel.	Day 92 Net. rel. *v.* August	Day 92 Net. rel. *v.* AS	Day 92 Spec. rel.
1 (Primed)	Liver membranes	100:1	10.70	5.63	5.07	9.82	4.08	5.74
		50:1	ND	ND	ND	3.59	0.90	2.69
		25:1	ND	ND	ND	1.51	0.59	0.92
2 (Primed)	Spleen and lymph node membranes	100:1	11.81	8.70	3.11*	8.87	7.16	1.71
		50:1	8.29	5.55	2.74	ND	ND	ND
		25:1	4.24	3.71	0.53	ND	ND	ND
3 (Unprimed)	Spleen and lymph node membranes	100:1	2.87	5.07	−2.20	1.23	2.95	−1.72
		50:1	2.99	1.85	1.14	ND	ND	ND
		25:1	0.21	2.64	−2.43	ND	ND	ND
4 (Primed)	Spleen and lymph node whole cells (2,500 R)	100:1	19.59	5.51	14.08†	34.06	8.00	26.06†
		50:1	11.96	3.96	8.00†	21.85	6.61	15.24†
		25:1	6.31	2.81	3.50	15.97	5.53	10.44†

Primed AS rats were given 2×10^7 August lymphoid cells, i.p. on day −120. Groups 1, 2 and 3 were given August cell membranes approximately equal to 3.5×10^7 lymphoid cells, i.v. on days 0, 21, 36, 57, 71 and 85. Group 4 was given 3.5×10^7 August lymphoid cells (2,500 R), i.v. on days 0, 21, 36, 57, 71 and 85. Peritoneal exudate lymphocytes taken from injected rats, on the days indicated, were tested for lysis of ^{51}Cr-August and ^{51}Cr-AS (syngeneic) targets. Spontaneous release (already subtracted) varied between 6 and 14% of the total count. Results are expressed as means of triplicates. Significant specific release results are indicated.
*$P < 0.05$, †$P < 0.01$.

Table 3 Primary antibody and CMI responses after injection with viable allogeneic cells containing different amounts of Ia antigens

Injected cells	Ia content	Primary antibody response	Primary CMI response
Spleen cells	+++	+++	+++
Fc receptor-negative T cells	−	++	ND
Fibroblasts	±	+	+
Spermatozoa	±	+	++
EL4	−	+++	+++

AS strain rats were injected with August strain tissues shown, except in the case of EL4, where cultured mouse tumour cells ($H\text{-}2^b$) were injected into DBA/2 ($H\text{-}2^d$) mice. Fc receptor-negative T cells were prepared by passage of nylon-wool and G10 column purified T cells over IgG anti IgG columns. Fibroblasts were used after 8–12 subcultures; the presence of some contaminating macrophages was not excluded and therefore the presence of Ia is scored as ±.

ponses against Ia-like determinants. The presence of a second epitope alone (Ia, DNP, BSA) does not provide the additional signal, whereas intact lymphocytes do. Indeed, intact lymphocytes may provide the necessary signal to endowing amounts of any non-self cell-bound component with powerful immunogenicity.

Like allogeneic platelets, liver membranes provoked secondary antibody responses against Ag-B, but not Ia-like determinants. Spleen and lymph-node cell membranes, and Ag-B plus Ia-bearing liposomes induced secondary antibody responses against both epitopes. Secondary antibody responses therefore seem to be independent of the carrier, a fact likely to be of practical importance in clinical transplantation.

Surprisingly in view of the *in vitro* data[18], the secondary cellular immune response *in vivo* does seem to depend on the carrier. In spite of repeated boosts, minimal CMI was induced except by heavily irradiated, intact lymphocytes.

Our experiments show therefore that induction of primary immune responses (humoral and cellular) against MHC antigens *in vivo* depends both on the presentation of alloantigen and on a second signal. Without this, the small quantities of MHC antigen present on conventional allografts would not induce primary immunity. The second signal is not simply a second epitope, such as Ia. It is provided by intact, viable lymphocytes which need not be capable of division. Induction of secondary humoral responses does not require the second signal, the presence of alloantigen only being sufficient.

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Ionic channel formation in a living cell membrane

There have been several attempts to study cell membrane formation by the use of protoplasmic droplets extruded from plant cells into appropriate solutions[1]. The formation of a membrane on the surface of such droplets has been confirmed by optical, electrical and other measurements[2–4], but some properties of these unnatural membranes have been questioned[5]. I report here experiments which indicate the possibility of observing membrane formation in a living cell. The method was based on the sealing effect[6–8] which isolates a microelectrode tip from protoplasm into which it has been inserted.

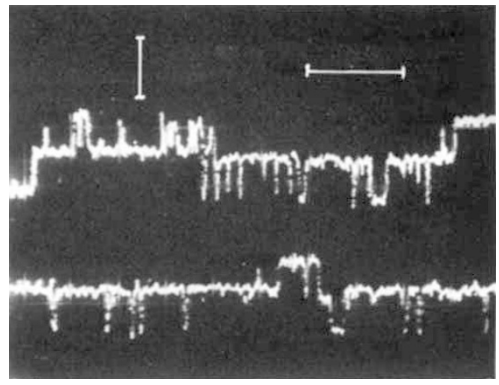

Fig. 1 Record of current obtained with a membrane containing two channels. The lowest part of each trace indicates the level of zero current. Membrane potential was −75 mV. The test microelectrode contained 100 mM LiCl, 2 mM $CaCl_2$. Horizontal bar represents 0.2 s, vertical bar 4×10^{-12} A.

Single cells of the freshwater alga *Nitellopsis obtusa* were used. A small portion of the cell was voltage clamped in an arrangement similar to that of the 'double sucrose gap' technique[9]. The potential of the plasmalemma was controlled by means of a glass microelectrode filled with 1 M KCl connected to the clamp amplifier. This microelectrode did not usually seal during the several hours needed for the experiment to be completed. Another microelectrode (to be sealed) with a very thin tip (about 1 μm across) was inserted not more than 10 μm into the protoplasm. In most cases this test microelectrode was filled with one of the following solutions: 100 mM KCl, 2 mM $CaCl_2$; 100 mM NaCl, 2 mM $CaCl_2$; 100 mM LiCl, 2 mM $CaCl_2$; 50 mM $CaCl_2$. After the test microelectrode had become sealed, its potential was small compared with the previously measured resting potential, varying from −5 mV to −15 mV. This new test microelectrode potential was clamped by means of an additional feedback amplifier (feedback resistance was equal to 3×10^9 Ω). The changes in the current flowing through the test microelectrode occurred as voltage impulses at the output of this amplifier, and were recorded at different plasma-

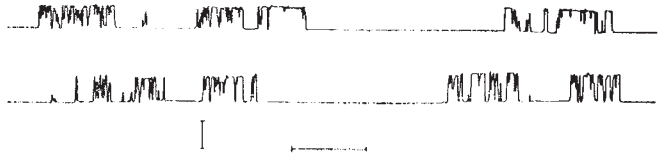

Fig. 2 Records of current showing inactivation intervals. One channel was present. Current did not always reach maximum value because of the limited response speed of the pen recorder. Upper record, membrane potential −75 mV. Lower record, membrane potential −65 mV. Test microelectrode solution: 50 mM KCl, 50 mM NaCl, 2 mM CaCl. Horizontal bar represents 6 s, vertical bar 10^{-12} A.

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