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Thymus-Derived Rat Lymphocyte Receptor for Cell Surface Antigens Is a Nonserologically Defined Product of the Major Histocompatibility Gene Complex

(cell receptors/antigen recognition/alloantigens)

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ABSTRACT **Recognition of cell surface antigens by nonsensitized thymus-derived rat lymphocytes is not affected by antisera against immunoglobulin, yet can be effectively blocked by treatment of the lymphocytes with alloantisera raised against lymphoid cells. However, alloantisera induced by nonlymphoid cells do not inhibit recognition. Adsorption of blocking antisera on immunoglobulin-Sepharose columns does not decrease their blocking activity. Absorption of blocking alloantisera with liver and kidney homogenates removes the cytotoxic alloantibodies, yet does not affect the capacity of the antisera to block recognition. Anti-Hl-1 alloantisera block antigen recognition only of lymphocytes of strains that share the Hl-1 locus. These results suggest that the constant part of the thymus-derived lymphocyte receptor for cell-surface antigens is a product of the rat major histocompatibility locus, which is not identical with the serologically defined antigens.**

Bone marrow-derived (B) lymphocytes have been shown to constitute a diverse population with regard to their receptors for antigens (1–3). Furthermore, binding of antigens to B lymphocytes was shown to be a "passive" process, and the binding specificity was found to be similar to that manifested by the antibodies produced by these cells (4). These observations, and the blocking effect of antibodies against immunoglobulin on antigen recognition (5), have made it generally accepted that the cell receptor for antigens on B cells is an immunoglobulin molecule. A question that remained open, however, is the nature of the cell receptors of thymus-derived lymphocytes (T cells), which are the effector cells in cell-mediated immunity and which participate as helpers to B lymphocytes in the production of antibodies.

Using fibroblasts as cellular immunoadsorbents, we demonstrated that the specificity of cell-mediated immunity induced *in vitro* is based on a state of diversity of T lymphocytes with regard to their receptors for the surface antigens of the fibroblasts (6). Recognition, i.e., binding of lymphocytes to the surface antigens of the fibroblasts, was found to be an active process in this system: it took place only at physiological temperature and was preventable by metabolic inhibitors (7). This was deduced from experiments in which rat lymphocytes were incubated on mouse fibroblast monolayers of a given H-2 phenotype. After an incubation period of between 30 and 120 min, 3–4% of the cells adhered to the monolayer. The lymphocytes that did not adhere were separated from those adhering and were transferred to a fresh monolayer, syngeneic to the first one. Both the adherent and nonadherent groups were cultured for 4–5 days for sensitization, then tested for their capacity to lyse specifically ^{51}Cr-labeled target cells syngeneic to the sensitizing monolayer (8). We demonstrated that the lymphocytes that did adhere initially underwent blast transformation and developed a high degree of lytic activity towards the target fibroblasts. The lymphocytes that did not adhere to the initial monolayer and were transferred for sensitization to a fresh monolayer acquired a considerably lower cytolytic activity. These nonadherent lymphocytes could, however, develop specific cytotoxicity when sensitized on a monolayer of unrelated H-2 phenotype. Hence, absorption is antigenically specific, leading to a depletion from the cell population of those lymphocytes which possess receptors for the surface antigens of the adsorbing fibroblasts. Specific lymphocyte adherence thus reflects the process of recognition.

With the aim of characterizing the receptors of T lymphocytes that recognize the surface antigens of the fibroblasts, we applied, in the present investigation, antisera against various lymphocyte membrane components which might themselves constitute the cell receptors.

Abbreviations: T and B lymphocytes, thymus-derived and bone marrow-derived lymphocytes, respectively; H- and L-chains, heavy and light chains, respectively.

MATERIALS AND METHODS

Animals. Female Lewis and BN rats, 2–6 months of age, from our local breeding center, were used, as well as rats of strains Lewis, BS, AS, L·AS2, L·BN, and (Lewis × BN)F_1 from the Max-Planck-Institut für Immunobiologie at Freiburg. Cultures of fibroblasts were prepared from different rat strains and from C3H/eb and Balb/c mice.

Assay of Recognition of Cell-Surface Antigens by T Lymphocytes. All the cell culture methods have been extensively described (7, 8). Briefly, 50 × 10^6 normal rat lymph node cells were incubated on secondary cultures of mouse fibroblasts in 2 ml of culture medium (85% Eagle's medium and 15% horse serum) (8). After an incubation time of 1–2 hr, the nonadherent lymphocytes were separated from the lymphocytes that adhered to the fibroblasts (7) and transferred to fresh cultures (30 × 10^6 nonadhering lymphocytes in 4 ml of medium). The cultures containing the adhering lymphocytes were immediately replenished with 4 ml of culture medium per plate. All the culture types were incubated for an additional period of 4 days to permit sensitization of

TABLE 1. *Effect of antibodies against immunoglobulin on recognition of cellular antigen by T lymphocytes*

Exp. no.	Treatment*	% Cytotoxicity: Adhering	% Cytotoxicity: Nonadhering	Coefficient of adherence†	% Inhibition‡
1	Control	14.3	7.0	2.05	—
	L_1 (0.5 ml)	13.3	4.5	2.96	0
	L_1 (1.5 ml)	16.3	4.0	4.06	0
	L_2 (0.5 ml)	13.5	6.3	2.14	0
	L_2 (1.5 ml)	12.3	6.0	2.05	0
	L_3 (1.5 ml)	19.4	3.8	5.10	0
2	Control	33.2	16.4	2.02	—
	L_3 (6 hr)	39.6	7.5	5.25	0
	L_3 (3 days)	23.5	13.2	1.78	23
3	Control	28.5	13.8	2.06	—
	H-(Fab)′	23.6	9.4	2.51	0
	L-(Fab)′	31.1	11.6	2.68	0
4	Control (N)	52.1	25.6	2.04	—
	H-(Fab)′ (N)	47.3	24.4	1.94	9
	L-(Fab)′ (N)	48.3	27.8	1.74	30

* L_1, L_2, and L_3, different antisera against L-chain; L-(Fab)′ and H-(Fab)′, (Fab)′ fragments of antisera against L- and H-chain, respectively; (N), neuraminidase pretreatment of the lymphocytes.

† Coefficient of adherence (CA) = (% cytotoxicity of adhering cells)/(% cytotoxicity of nonadhering cells).

‡ % Inhibition of recognition = [CA(untreated) − CA (treated)]/[CA(untreated) − 1].

the lymphocytes by the fibroblast antigens. Then 3×10^6 living sensitized cells were transferred to ^{51}Cr-labeled target fibroblast cultures (8) to measure the extent of lysis. Recognition is expressed quantitatively in the coefficient of adherence, which is the cytotoxicity manifested after sensitization of the adherent cells, divided by the cytotoxicity obtained after sensitization of the nonadherent cells.

Demonstration of Binding of Antibodies against Immunoglobulin to Lymphocytes. To test whether our preparation of antibodies against immunoglobulin binds to lymphocytes (presumably B cells), we applied an immunofluorescence sandwich technique. Lewis rat lymph node lymphocytes were incubated with antisera against immunoglobulin. The cells were washed, and 10 mM sodium azide was added. Subsequently, fluorescein-labeled antiserum against rabbit immunoglobulin prepared in goat was added, the cells were again washed in the cold, and the preparation was screened for fluorescence under a Zeiss fluorescence microscope.

Antisera against L- and H-Chains. For the preparation of L (light)- and H (heavy)-chains, the IgG fraction was isolated from normal Lewis rat serum as described above. The single chains were prepared by reduction in Clelant's reagent, alkylation with iodoacetamide, and fractionation on a Sephadex G-100 column. The preparations were tested for purity by polyacrylamide electrophoresis.

Five rabbits were immunized against L-chains and five against H-chains by twice injecting 2 mg of protein in complete Freund's adjuvant, with an interval of 2 weeks between injections. The animals were bled 2 weeks after the second injection and the sera were tested for anti-Ig activity in an Ouchterlony test.

Monovalent (Fab)′ fragments were prepared by the method of Inbar *et al.* (9).

Alloantisera. Anti-lymphocyte alloantisera were produced by intradermal injection of BN rats with 5×10^8 Lewis rat spleen and lymph node cells immersed in complete Freund's adjuvant, followed 14 days later by intraperitoneal injection of 3×10^8 Lewis lymphocytes. After a further period of 8 days, the animals were bled and their sera were heat-inactivated.

Anti-skin alloantisera were prepared by grafting Lewis skin epithelium onto BN recipients and regrafting 12 days after rejection of the first graft. The rats were bled 1 day after rejection of the second graft.

Animals immunized with lymphocytes showed second-set type rejection of Lewis test skin homografts.

Absorption of Alloantisera. The immunoglobulin fraction of normal Lewis serum was precipitated with 45% ammonium sulfate at 4° and dialyzed against phosphate-buffered saline. This fraction contained 70% 7S, 25% 17S, and 5% 11S globulins, as determined by ultracentrifugation. By the procedure of Givol *et al.* (10), 15 mg of Ig per g of Sepharose were coupled. By the same method, bovine serum albumin was conjugated to Sepharose. Volumes of 1.0 ml of antisera were absorbed on protein-Sepharose columns of a capacity of 2.5 ml. The antigen-absorbing capacity of Ig columns was tested with standard antisera against immunoglobulin and documented on Ouchterlony plates.

Alloantisera were absorbed on rat thymocytes by incubating 1 ml of antiserum with 10^9 (washed three times) thymocytes in phosphate-buffered saline at room temperature for 30 min, then at 0° for 30 min.

Alloantisera were absorbed on nonlymphoid tissues as follows. Donor animals were extensively bled and perfused with phosphate-buffered saline. The kidneys, livers, and brains were excised and homogenized in a Potter-Elvehjem homogenizer. The homogenates were washed eight times with phosphate-buffered saline until the supernatants were clear. One volume of antiserum diluted 1:5 was incubated with 1 volume of homogenate pellet for 30 min, at 4°, then centrifuged at $27,000 \times g$ at 0°.

Both absorbed and nonabsorbed sera were tested for humoral cytotoxic activity by the dye exclusion test, by the method of Boyse *et al.* (11).

RESULTS

Antibody against Immunoglobulin Does Not Affect Recognition. To characterize the T lymphocyte recognition structure, pretreatment of lymphocytes with antibodies against known surface antigens was attempted. An antibody directed against receptor determinants should bind to the receptor, preventing the "blindfolded" lymphocyte from recognizing the antigen.

It is generally accepted that the B lymphocyte receptor is a conventional membrane-bound immunoglobulin. We, therefore, investigated first the effect of different preparations of antibody against immunoglobulin on T lymphocyte recognition. We tested five different rabbit antisera against Lewis rat H- and L-chains or purified $(Fab)_1$ fragment preparations. Binding of antisera against immunoglobulin to lymphocytes was tested by indirect fluorescence staining. About 15–20% of the lymph node cells treated with antisera against L-chain were stained.

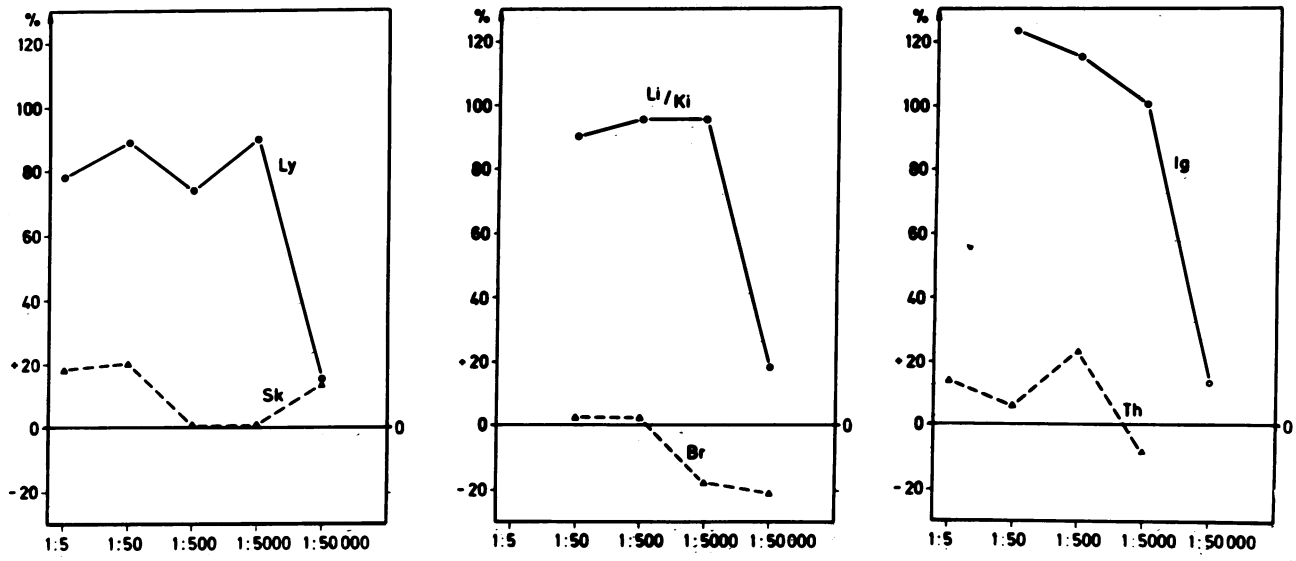

FIG. 1. Percent inhibition of recognition by different BN anti-Lewis alloantisera. Sk, BN-α-Lewis anti-skin alloantiserum; Ly, BN-α-Lewis anti-lymphocyte alloantiserum; Ig, BN-α-Lewis anti-lymphocyte alloantiserum absorbed on Ig-Sepharose; Th, BN-α-Lewis anti-lymphocyte alloantiserum absorbed on Lewis thymocytes; Li/Ki, BN-α-Lewis anti-lymphocyte alloantiserum absorbed on liver/kidney homogenates; Br, BN-α-Lewis anti-lymphocyte alloantiserum absorbed on brain homogenate. Ordinate, % inhibition of recognition; abscissa, dilution of alloantisera.

We tested the influence of different antisera against L-chain at different concentrations on recognition of mouse cellular antigen by Lewis lymphocytes. Equal numbers of lymph node cells (200 $\times$ 10^6 cells per group) were incubated for 30 min at 37° in 5-ml volumes of Eagle's medium containing either 1.5 ml of normal rabbit serum (for controls) or portions of either 0.5-ml or 1.5-ml volumes of different batches of rabbit antiserum against Lewis L-chain. As documented in Table 1, Exp. 1, in no case did pretreatment with any of the antisera decrease the coefficient of adherence. Thus, recognition was not inhibited by antibodies against immunoglobulin.

In other experiments (Table 1, Exp. 2), we tested whether recognition is affected by prolonged presence of the antisera. Instead of pretreating the lymphocytes before the recognition assay, we kept 0.5 ml of the antiserum in each culture plate during the coincubation phase as well as during the first 3 days of culture. This procedure did not yield significant decrease of the coefficient of adherence.

We previously reported that the T lymphocyte receptor is apparently partly hidden by the strong sialic acid coat characteristic of T lymphocytes and that treatment of the lymphocytes with neuraminidase exposes the receptor (7). It seemed possible that the antibodies against immunoglobulin could not bind to the receptor because of steric hindrance by carbohydrate moieties. Therefore, we pretreated lymphocytes with neuraminidase, followed by incubation with (Fab)' fragments of antibodies directed against H- or L-chains of Lewis Ig (450 μg/ml). Controls were similarly treated with antibodies, but without neuraminidase pretreatment. The results (Table 1, Exps. 3 and 4) demonstrate that neuraminidase treatment does not render lymphocytes susceptible to antibody.

Alloantisera Inhibit Recognition. The immune reactivity of the lymphocytes appears to be controlled by genes closely associated with the histocompatibility gene loci (12). Treatment of lymphocytes with alloantisera directed against the histocompatibility antigens was found to decrease their reactivity against allogeneic lymphocytes in a mixed lymphocyte reaction or their capacity to induce a graft versus host reaction (13–15). We treated Lewis lymphocytes with alloantisera raised in BN rats against Lewis lymphoid cells (anti-lymphocyte alloantisera). With five independent serum batches applied, we found strong inhibition of recognition even at very high dilutions. In no case could visible agglutination of the lymphocytes be observed at dilutions higher than 1:5. Fig. 1 (left) shows that a representative BN anti-Lewis lymphocyte alloantiserum with a cytotoxic titer of 1:2048 totally inhibited recognition of C3H/eb fibroblast antigens by Lewis lymphocytes up to a dilution of 1:5000.

To determine which antibody component is responsible for the blocking of recognition, we tested the effect of alloantisera produced by immunizing BN rats with nonlymphoid tissues. Alloantisera were produced by grafting Lewis skin epithelium onto BN recipients (anti-skin alloantiserum). Anti-skin alloantiserum with a cytotoxic titer of 1:2048 did not block recognition, even at the lowest dilutions.

Similar results were obtained with alloantiserum against Lewis sarcoma cells.

Absorption of Blocking Alloantisera on Different Immunoabsorbents. To test whether the blocking effect of anti-lymphocyte alloantiserum is due to antibody against cell-bound immunoglobulin, we absorbed the serum on an Ig-Sepharose column known to contain IgM, IgG, and IgA. The Ig-Sepharose column was tested for its capacity to absorb antibodies against rat Ig. We found that absorption on such a column neither decreased the blocking capacity of the serum nor lowered cytotoxic titers (Table 2 and Fig. 1, middle).

We examined the effect of absorption of the anti-lymphocyte alloantiserum on different Lewis rat tissues. Lewis thymocytes fully absorbed both the cytotoxic and the blocking activities. Absorption on brain homogenate yielded the same results. However, kidney and liver homogenates absorbed the cytotoxic activity but did not affect the blocking capacity (Table 2). Thus, the blocking antibody of anti-

TABLE 2. *Effect of absorption of anti-Lewis lymphocyte alloantiserum on blocking and cytotoxic activities*

Immunoabsorbent	Cytotoxic index* (%)	Blocking activity†
Ig-Sepharose	92	No absorption
BSA-Sepharose	100	Not done
BN thymocytes	96	No absorption
Lewis thymocytes	0	Full absorption
Kidney	0	No absorption
Liver	0	No absorption
Brain	5	Full absorption

BSA, bovine serum albumin.

* Percent cytotoxic activity compared to unabsorbed anti-lymphocyte alloantibodies, measured in dye exclusion tests. Serum dilution, 1:5.

† The results shown in Fig. 1 are included.

lymphocyte alloantisera appears to be directed against a lymphocyte alloantigen, which is identical neither to a classical immunoglobulin nor to a serologically defined antigen of the major histocompatibility complex.

To determine the genes controlling the receptor molecule, we treated lymphocytes of genetically different rats with blocking anti-Lewis alloantisera (anti-H1-l) prior to the recognition assay. Both unabsorbed alloantisera and alloantisera absorbed on liver cells were tested. The results were (Table 3, Exps. 1–3) that the alloantisera (anti-H1-l) blocked recognition of lymphocytes from rats of other strains (BS, AS) which share the H1-l genotype, whereas lymphocytes from H1-different rats, such as BN (H1-n) or DA (H1-a), were not blocked (unpublished results). Neither did these anti H1-l antisera affect recognition of lymphocytes from L·AS2 and L·BN rats, which are congenic with Lewis, differing only in the H1 locus (Table 3, Exps. 4 and 5). Lymphocytes of (Lewis × BN)F_1 hybrids were only marginally affected by the antisera (Table 3, Exp. 6).

DISCUSSION

When lymphocytes are treated with antibodies directed against a single known type of surface antigens, these antigens are induced to migrate horizontally within the cell membrane to form aggregates. However, the other unrelated antigens remain unaffected (16). Hence, it seems reasonable to assume that the loss of recognizing capacity after treatment with antibodies is indeed due to the blocking of the cell receptors rather than to a general perturbation of the cell membrane.

There are claims that T lymphocytes carry immunoglobulin on their surface even in amounts comparable to those of B lymphocytes (17). Yet, although we tested 10 different preparations of antibody against immunoglobulin under different conditions, in no case were we able to inhibit significantly T cell recognition by antibodies against immunoglobulin. Since it is possible that only antiidiotype antibodies block specific recognition (18) and that our batches of antiserum against immunoglobulin did not contain a sufficient amount of such antibodies, we absorbed anti-lymphocyte alloantisera on Ig-Sepharose columns known to contain IgM, IgG, and IgA. On the assumption that these immunoglobulins contain at least some amount of the critical T cell immunoglobulin idiotype, absorption of blocking sera should result at least in reduction of the blocking activity in the higher dilutions. Yet, we were unable to detect any decrease of blocking activity after absorption of anti-lymphocyte alloantisera on Ig-Sepharose columns. Hence, we conclude that the T cell receptor for cell-surface antigens cannot be a conventional immunoglobulin.

Alloantisera induced with lymphoid cells very effectively prevent T lymphocytes from recognizing foreign antigen. Several groups have found that treatment of lymphocytes with alloantisera inhibited their reactivity in mixed lymphocyte cultures (13, 14) and graft versus host reactions (15). Similarly, alloantisera inhibited binding of soluble antigens to T lymphocytes and stimulation of primed T cells by the anti-

TABLE 3. *Effect of anti-Lewis lymphocyte alloantisera, unabsorbed or absorbed on Lewis liver homogenate, on recognition of allogeneic fibroblasts by lymph node cells from different rats*

	Lymphocytes		Fibroblasts			% Specific ^{51}Cr release			
Exp.	Strain	H1	Strain	H1	Anti-sera*	Adhering	Nonadhering	CA†	% Inhibition of recognition
1	Lewis	l	L.AS2	f	ϕ	26.6	13.3	2.00	—
					L	18.3	14.2	1.28	72
					A	21.5	20.0	1.07	93
2	BS	l	L.BDV	d	ϕ	29.9	13.0	2.31	—
					L	21.1	23.8	0.89	100
					A	20.1	22.9	0.88	100
3	AS	l	BDV	d	ϕ	34.1	14.3	2.41	—
					L	18.2	16.8	1.13	91
					A	20.1	21.3	0.95	100
4	L.AS2	f	BDV	d	ϕ	30.8	12.1	2.51	—
					L	32.0	14.3	2.24	—
					A	28.1	13.2	2.15	25
5	L.BN	n	AS2	f	ϕ	42.3	15.8	2.68	—
					L	40.1	14.9	2.70	0
					A	43.4	14.0	3.10	−25
6	(Lew × BN)	l/n	AS2	f	ϕ	29.1	14.0	2.08	—
					L	28.5	16.6	1.75	30
					A	22.9	13.5	1.70	35

* ϕ, normal BN serum; L, BN-α-Lewis, nonabsorbed; A, BN-α-Lewis, absorbed on liver homogenate.

† CA, coefficient of adherence.

gen (19). Thus, serologically defined antigens of the histocompatibility locus appeared to be involved in antigen recognition. This possibility was ruled out by our experiments, because (*a*) alloantisera induced by nonlymphoid cells contained no blocking activity and (*b*) blocking antisera that were absorbed on kidney and liver homogenates lost all their complement-dependent cytotoxicity but none of their blocking activity. Analogous results were recently obtained in other T cell-dependent systems. *In vitro* formation of anti-sheep erythrocyte plaque-forming cells was blocked by liver-absorbed anti-lymphocyte alloantibodies (B. Bhakdi, personal communication). The same sera inhibited concanavalin A stimulation of Lewis lymphocytes (H.W., unpublished results). It, therefore, appears that alloantisera which block T lymphocyte recognition contain a noncytotoxic antibody directed against the T lymphocyte receptor, and that this receptor is unrelated to the serologically defined specificities of the H-antigens.

The finding that T cell recognition cannot be blocked by alloantisera elicited by nonlymphoid tissues corroborates this conclusion. Moreover, it argues against a close physical association between the T cell receptor and serologically defined antigens, as suggested by Shevach *et al.* (20).

The possible participation of serologically defined specificity in antigen recognition seems to have been suggested by the observation that sera directed against β_2-microglobulin inhibited human mixed lymphocyte reactions (21). Since the β_2-microglobulin was found to be a constant moiety of the HL-A antigen (22), one could assume that the HL-A is associated with the receptor. However, immunofluorescence studies have indicated that β_2-microglobulin not associated with HL-A exists on the cell membrane. It is the latter that might be related to the receptor site.

Experiments carried out in our study to determine the genetic control of the T cell receptor indicated that anti-Lewis alloantisera blocked recognition of lymphocytes of different genetic origin which share the H1-l genotype. Lymphocytes of different H1 genotypes, or lymphocytes congenic with Lewis rat, yet different in the H1 locus, were not blocked by these antisera. Hence, we conclude that the T cell receptor is an alloantigen determined by the H1-l gene. This rat T cell receptor seems analogous to membrane products of the Ir region of the murine H–L complex. This chromosome section controls immunoreactivity of T cells and determines nonserologically defined membrane antigens.

The blocking antibodies effective in our alloantisera are strain-specific and show no specificity with respect to the recognized antigen. These antibodies must, therefore, be directed against the constant part rather than against the antigen-specific combining site of the receptor molecule.

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