Immunology 1984 **53** 79

Inhibition of autologous mixed lymphocyte reaction by monoclonal antibodies specific for the β chain of HLA-DR antigens

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Accepted for publication 2 April 1984

Summary. Recent studies using rabbit antisera to the separated HLA-DR α and β subunits have suggested that α chain-specific, but not β chain-specific, antisera inhibit T cell proliferative responses in primary and secondary human autologous mixed lymphocyte reaction (AMLR).

In the present study, with the aid of sequential co-precipitation assays and Western blotting methods, a monoclonal rat alloantibody 1E4, specific for the β chain of rat class II molecules carrying an Ia determinant Ba-2·7, was characterized to recognize a monomorphic determinant located on the β chain of DR antigens. This antibody and a murine monoclonal antibody HU-4, also specific for the β chain of DR antigens, strongly inhibited both primary and secondary AMLR through a mechanism distinct from an antibody-dependent cell-mediated cytotoxicity reaction. These results indicate that the inhibition of AMLR is not a unique feature of DR α-specific antibodies.

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INTRODUCTION

Normal human T cells proliferate *in vitro* when cocultured with autologous non-T cells (Opelz *et al.*, 1975; Kuntz, Innes & Weksler, 1976). This reaction, termed the autologous mixed lymphocyte reaction (AMLR), has memory and specificity (Weksler & Kozak, 1977), and is thought to represent a self-recognitive mechanism that might be important in regulating the cellular interactions involved in the generation of normal immune response.

In this reaction, HLA-D region antigens including HLA-DR have been proposed as one of the stimulating antigens, because polyclonal alloantisera and monoclonal antibodies (MoAbs) directed against DR antigens inhibit proliferative responses of T cells to autologous non-T cells (Bergholtz, Albrechtsen & Thorsby, 1977; Gottlieb *et al.*, 1979; Huber *et al.*, 1981; Palacios & Möller, 1981; Mingari & Moretta, 1982). In a study to determine the role of individual chains of DR antigens in AMLR, Palacios *et al.* (1982) found α chain-specific, but not β chain-specific, xenoantiserum to inhibit T cell proliferative responses in primary and secondary AMLR, suggesting a specific role for the α chain in T cell proliferations in this reaction.

In the present study, we demonstrate that, in contrast to their results, two MoAbs specific for the β chain of DR antigens can inhibit both primary and secondary AMLR, suggesting that the inhibition of

AMLR is not a unique feature of DR α-specific antibodies.

MATERIALS AND METHODS

Antibodies

Four MoAbs 1E4, HU-20, HU-4, and HU-2 were used in the present study. They were prepared and characterized as previously described (Koide *et al.*, 1982; Kasahara *et al.*, 1983; Natori *et al.*, 1983; Ikeda *et al.*, 1984). Briefly, 1E4 (IgG2b) was obtained by fusing murine myeloma cells P3−X63-Ag8 with splenocytes from ACI (*RT1^a*) rats immunized with spleen and lymph node cells from WKA (*RT1^k*) rats (Natori *et al.*, 1983). This antibody recognizes the β chain of rat class II molecules carrying an Ia determinant Ba-2·7 and cross-reacts with all human B cells. The remaining three MoAbs HU-20 (IgG2), HU-4 (IgG2) and HU-2 (IgG) were produced by immunizing BALB/c mice with EB virus-transformed human B lymphoid cell lines. Out of them, HU-20 (previously denoted 4G6) and HU-4 (previously denoted 3G7) have been characterized to recognize monomorphic determinants on DR, but not on DC or other class II, molecules on the basis of extensive sequential co-precipitation (Koide *et al.*, 1982; Kasahara *et al.*, 1983) and two-dimensional gel experiments (Ikeda *et al.*, 1984). HU-2 recognizes a framework determinant on HLA-A, -B and -C molecules.

A human alloantiserum KY22 with DC1 (=MB1) specificity was obtained from a renal allograft recipient (kindly provided by Drs T. Oka and Y. Ohmori, Kyoto Prefectural University of Medicine, Kyoto, Japan); this antiserum contains anti-DC1 antibodies, but is devoid of anti-DR2 antibodies as previously described (Koide *et al.*, 1982).

To test the capacity of MoAbs to inhibit T cell proliferative responses in AMLR, immunoglobulins were enriched from ascitic fluids by fractionation on Sephadex G-200 or Protein A-Sepharose 6MB columns (Pharmacia Fine Chemicals, Uppsala, Sweden).

Preparation of F(ab′)$_2$ fragments

F(ab′)$_2$ fragments of the MoAb 1E4 were prepared by treating the IgG fraction purified with a Sephadex G-200 column with pepsin (P-L Biochemicals Inc., Milwaukee, WI, U.S.A.), according to the method of Nisonoff, Wissler & Woernley (1960). After cleavage, the fractions containing F(ab′)$_2$ were separated by applying the pepsin-digested material to a Sephadex G-150 (superfine) column (Pharmacia Fine Chemicals, Uppsala, Sweden). The purity of the F(ab′)$_2$ fraction, as determined by its electrophoretic pattern on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), was more than 90%.

Partial purification and labeling of B lymphoid cell glycoproteins

B lymphoid cell glycoproteins were partially purified from an EB-virus-transformed human B lymphoid cell line EBV-HO (*HLA-Aw24, Bw52, Dw12, DR2, DC1* homozygous). Briefly, 1 g (wet weight) of EBV-HO cells were solubilized with 10% Brij in phosphate-buffered saline (PBS) containing 1 mM phenylmethylsulphonyl fluoride (Aldrich Chemical Co. Inc., Milwaukee, WI, U.S.A.). The solubilized cells were then centrifuged at 10,000 *g* for 30 min, and the resultant supernatant was purified by affinity chromatography on *Lens culinaris* haemagglutinin coupled with Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). The glycoprotein fraction thus obtained was radiolabelled with ^{125}I by the chloramine-T method as described by Greenwood, Hunter & Glover (1963). The radiolabelled material is referred to as ^{125}I-EBV-HO.

Sequential co-precipitation assays

Sequential co-precipitation assays were performed essentially as described elsewhere (Katagiri *et al.*, 1979). Briefly, ^{125}I-EBV-HO (about 100,000 c.p.m.) was incubated with the first antibody overnight at room temperature. After further incubation with goat anti-human Ig, rabbit anti-mouse Ig or rabbit anti-rat Ig serum for 1 hr, the resultant precipitate was removed by centrifugation. The supernatant was subsequently tested by direct binding assays for the remaining binding activity with various antibodies including those used in the first binding reaction. The extent of binding was expressed as the percentage of radioactivity added, and % specific binding was calculated as:

$$\frac{(\%\text{ binding by test antibody} - \%\text{ binding by normal serum})}{(100 - \%\text{ binding by normal serum})} \times 100$$

As a source of normal serum, normal sera from humans, rats, and mice were used.

Subunit localization of the determinants recognized by the MoAbs 1E4 and HU-4 with Western blotting

The EBV-HO cell glycoprotein preparation (about 10 μg), suspended in 0·05 ml of SDS sample buffer

containing 62·5 mM Tris-HCl (pH 6·8), 10% glycerol, and 1% SDS, was heated for 30 min at 45° or for 2 min at 100°, and subjected to 10% SDS-PAGE under non-reducing conditions as described by Johnson *et al.* (1982). The separated proteins were then electrophoretically transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Richmond, CA, U.S.A.) according to the method of Towbin, Staehelin & Gordon (1979), with minor modifications. The membrane was then saturated with 3% bovine serum albumin in PBS overnight at 4°. After six washes with PBS, the blocked membrane was cut into strips and incubated overnight at 4° with 1E4 or HU-4 in the ascites form at a final dilution of 1:100. The blots were then washed with six changes of PBS and incubated overnight at 4° with 1:250 diluted horseradish peroxidase (HRP)-conjugated goat anti-rat or mouse IgG (E-Y Laboratories Inc., San Mateo, CA, U.S.A.). For the colour reaction, the blots were soaked in a solution of 25 μg of o-dianisidine per ml/0·01% H_2O_2/10 mM Tris-HCl (pH 7·4) for 30 min at room temperature. The reaction was terminated by washing with PBS, and the blots were dried.

Mononuclear cell isolation and separation of T and non-T cells

Peripheral blood mononuclear cells from healthy adult volunteers were obtained by centrifugation of heparinized blood over the Ficoll-Conray gradient as described previously (Tomonari, 1980; Tomonari, Wakisaka & Aizawa, 1980). After depletion of adherent cells by incubation in 100 mm polystyrene petri-dishes (Falcon Plastics, Oxnard, CA, U.S.A.) at 37° in a humidified 5% CO_2 atmosphere for 1 hr, non-adherent cells recovered were separated into T and non-T cells by rosette formation with neuraminidase-treated sheep erythrocytes (SRBC) according to the procedure previously described (Tomonari, 1980; Tomonari *et al.*, 1980). After lysis of SRBC, T cells and non-T cells were washed twice with Hanks' balanced salt solution and resuspended in medium RPMI-1640 (Grand Island Biological Co., Grand Island, NY, U.S.A.) supplemented with 10 or 20% autologous fresh plasma, 2 mM L-glutamine, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES), and antibiotics.

Primary and secondary AMLR

Full details of the procedure have been described previously (Tomonari, 1980; Tomonari *et al.*, 1980). Briefly, 2×10^5 T cells and an equal number of non-T cells were cultured in triplicate in flat-bottomed microtitre plates (Flow Laboratories Inc., Hamden, CT, U.S.A.) in a total volume of 0·2 ml of medium RPMI-1640 supplemented with 20% autologous fresh plasma, 2 mM L-glutamine, 25 mM HEPES, and antibiotics. Before culture, non-T cells were treated with mitomycin C (Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan) at a concentration of 25 μg/ml for 30 min at 37°. Cultures were incubated at 37° in a humidified 5% CO_2 atmosphere for 7 days. Tritiated thymidine (1 μCi/well; NET-027, New England Nuclear, Boston, MA, U.S.A.) was added 8 hr before the harvest.

Secondary AMLR cultures were established by coculturing 5×10^4 viable cells recovered from 11-day primary AMLR cultures with 1×10^5 mitomycin C-treated non-T cells in flat-bottomed microtitre plates in a total volume of 0·2 ml of medium RPMI-1640 supplemented with 10% autologous fresh plasma, 2 mM L-glutamine, 25 mM HEPES, and antibiotics. Following 4 days of culture at 37° in a humidified 5% CO_2 atmosphere, tritiated thymidine (1 μCi/well) was added and cells were harvested 8 hr later.

When AMLR cultures were performed in the presence of MoAbs, each antibody (50 μl/well) at various concentrations was added at the initiation of the culture and left throughout the incubation period, unless otherwise specified.

RESULTS

Sequential co-precipitation analyses show that 1E4 preferentially reacts with DR molecules

The monoclonal rat alloantibody 1E4, specific for the β chain of rat class II molecules carrying an Ia determinant Ba-2·7 (Ohhashi *et al.*, 1981), cross-reacts with all human B cells, and recognizes a monomorphic determinant on human Ia antigens consisting of two components of mol. wts 32,000 (α chain) and 28,000 (β chain) under reducing conditions (Natori *et al.*, 1983). Because recent evidence indicates the existence of at least three distinct human Ia subsets DR, DC (=MB) and SB, it was considered important to identify 1E4-reactive Ia subsets. For this purpose, we performed sequential co-precipitation experiments using well-characterized antibodies specific for DR and DC as reference reagents. As illustrated in Fig. 1a, pretreatment of ^{125}I-EBV-HO with HU-20 (specific for DR) markedly decreased subsequent binding levels of

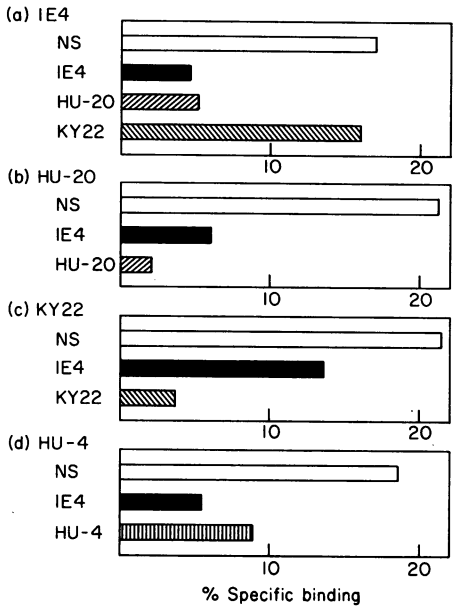

Figure 1. Sequential co-precipitation analyses of antigen molecules recognized by the MoAb 1E4. The radiolabelled antigen preparation ^{125}I-EBV-HO was first immunoprecipitated with NS (normal serum), HU-20, KY22, or HU-4 by using a predetermined amount of appropriate second antibodies (goat anti-human Ig, rabbit anti-mouse Ig, or rabbit anti-rat Ig). The resultant supernatant was subsequently assayed for the remaining binding activity with 1E4 (a), HU-20 (b), KY22 (c), and HU-4 (d). % Specific binding was calculated as described in 'Materials and Methods.' As a source of NS, normal sera from humans, rats, and mice were used. Because all of them gave virtually identical results, only the results obtained by normal rat serum are presented.

1E4 as compared with pretreatment with normal serum. Likewise, in experiments in the reciprocal order, the removal of 1E4-reactive molecules significantly reduced antigen molecules reacting with HU-20 (Fig. 1b) and HU-4 (specific for DR; Fig. 1d), suggesting that 1E4 preferentially recognizes an antigenic determinant carried on DR molecules. In contrast, depletion of Ia molecules from ^{125}I-EBV-HO with KY22 (specific for DC1) did not affect subsequent binding levels of 1E4 (Fig. 1a). Similarly, immunodepletion with 1E4 was not able to remove antigen molecules reacting with KY22 to a significant degree (Fig. 1c). Thus, these results indicate that the MoAb 1E4 preferentially reacts with DR, but not with DC, molecules.

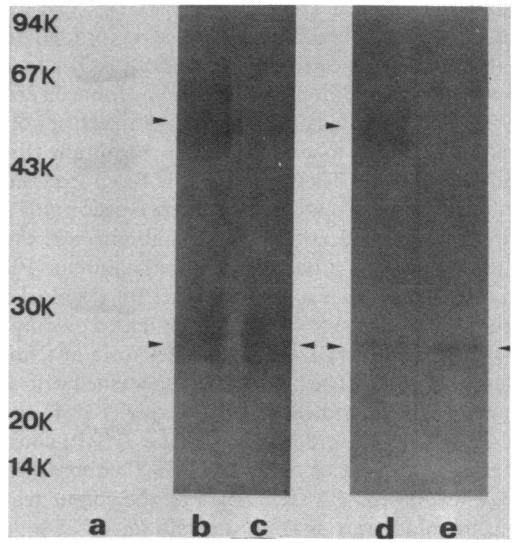

Figure 2. Subunit localization of the determinants recognized by 1E4 and HU-4. The unlabeled EBV-HO cell glycoprotein preparation was electrophoresed under non-reducing conditions in 10% SDS-PAGE after being heated either for 30 min at 45° (tracks b and d) or for 2 min at 100° (tracks c and e). The separated proteins were then electrophoretically transferred to a nitrocellulose membrane, and incubated with 1E4 (tracks b and c) or HU-4 (tracks d and e). The blots were detected by using HRP-conjugated goat anti-rat or mouse IgG as described in 'Materials and Methods'. In tracks (b) and (d), the separated β chain and the undissociated α–β complex of HLA-DR antigens are indicated by *arrows*. The *arrows* in tracks c and e indicate the location of the separated β chain. Track (a) gives molecular weight markers transferred to a nitrocellulose membrane and stained with Amidoblack 10B: phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400). The numbers on the ordinate represent the migration of the marker proteins; K = 1000.

Subunit localization of the antigenic determinants recognized by the MoAbs 1E4 and HU-4

To localize the antigenic determinants recognized by the MoAbs 1E4 and HU-4 on the subunit level of DR antigens, glycoproteins were isolated from EBV-HO cells, and the subunits were electrophoretically separated, transferred to nitrocellulose, and tested for reactivity with the MoAbs. The blots were detected by using HRP-conjugated goat anti-rat or mouse IgG, followed by colour reaction with o-dianisidine. Location of the α–β complex and the separated individual chains was determined by running molecular weight

marker proteins on the same gel (Fig. 2, track a). In the glycoprotein preparation containing 1% SDS and heated for 30 min at 45°, two major bands of 60,000 (α–β complex) and 28,000 (β chain) were obtained (Fig. 2, tracks b and d). In contrast, when the glycoprotein preparation was heated for 2 min at 100° (Fig. 2, tracks c and e), binding of 1E4 and HU-4 was restricted to the lower molecular weight bands in a position corresponding to the β chain, suggesting that complete dissociation of bimolecular complexes had taken place under these conditions. Similar binding patterns were obtained by testing 1E4 and HU-4 against six additional human B cell lines expressing different DR phenotypes (data not shown). Thus, we conclude that both 1E4 and HU-4 recognize antigenic determinants located on the β chain of DR antigens.

Inhibition of AMLR by 1E4 and HU-4

Addition of a final concentration of 0·2 μg/ml or more of the MoAb 1E4 or HU-4 without complement at the initiation of the cultures almost completely inhibited proliferative responses of T cells to autologous non-T cells (Table 1). In contrast, the MoAb HU-2 to a monomorphic determinant of HLA-A, -B and -C antigens had no effect even when added in high concentration. Kinetic experiments in which 1E4 was added on different days after the onset of the culture showed that 1E4 could induce significant suppression when added within 3 days, but was ineffective when added on day 5 of the culture (Table 2). Similar results were obtained in kinetic experiments using HU-4 (data not shown).

In the next step, in order to examine whether 1E4 and HU-4 can also inhibit secondary AMLR, T cells cultured with autologous non-T cells for 11 days were stimulated with freshly isolated autologous non-T cells in the presence of MoAbs (Table 3). Here also, 1E4 and HU-4 added at a final concentration of 0·2 μg/ml or more almost completely eliminated proliferative responses of T cells.

Although HU-4 has already been shown not to mediate an antibody-dependent cell-mediated cyto-

Table 1. Inhibitory effect of 1E4 and HU-4 on primary AMLR response*

MoAb	Final concentration of MoAb added (μg/ml)	Δc.p.m. ± SEM†	% Inhibition‡
Medium§	—	46,148 ± 883	0·0
HU-2	2·0	44,840 ± 4414	2·8
1E4	2·0	0 ± 0	100·0
1E4	0·2	14,443 ± 2265	68·7
1E4	0·02	44,126 ± 14,390	4·4
1E4	0·002	46,059 ± 8416	0·2
HU-4	2·0	0 ± 0	100·0
HU-4	0·2	14,032 ± 4610	69·6
HU-4	0·02	32,295 ± 896	30·0
HU-4	0·002	27,181 ± 2526	41·1

* AMLR cultures were established by culturing 2×10^5 T cells with 2×10^5 mitomycin C-treated non-T cells in the presence or absence of MoAb. Triplicate cultures were incubated at 37° for 7 days and proliferation was determined by the tritiated thymidine uptake during the last 8 hr of the culture period. T cells alone, 2181 ± 96; non-T cells alone, 800 ± 202.

† All data are expressed as mean Δc.p.m. (c.p.m. obtained when T cells were cocultured with autologous non-T cells minus c.p.m. with T cells alone) ± standard error.

‡ % Inhibition was calculated by dividing the mean Δc.p.m. in the presence of MoAb by the mean Δc.p.m. in the presence of control medium, subtracting the ratio from 1 and multiplying by 100.

§ RPMI-1640 supplemented with 20% autologous fresh plasma, 2 mM L-glutamine, 25 mM HEPES, and antibiotics.

Table 2. Kinetic study on the inhibitory effect of 1E4 on primary AMLR response*

Time of addition of 1E4 (days)	Δc.p.m. ± SEM†	% Inhibition‡
Medium§	15,334 ± 3048	0·0
0	105 ± 29	99·8
1	1136 ± 537	92·6
2	6412 ± 1475	58·2
3	4859 ± 1320	68·3
5	11,255 ± 283	26·6

* AMLR cultures were established as described in Table 1. 50 μl of 1E4 at a final concentration of 2 μg/ml were added on days indicated. T cells alone, 791 ± 111; non-T cells alone, 848 ± 168.

†‡§ See corresponding footnotes of Table 1.

toxicity (ADCC) reaction (Koide *et al.*, 1982), it is possible that 1E4 exerts its inhibitory activity by mediating an ADCC reaction. To exclude this possibility, $F(ab')_2$ fragments were prepared from 1E4 and their effects on primary AMLR were examined. As shown in Table 4, significant inhibition was observed when the $F(ab')_2$ fragments were added at a final concentration of more than 0·2 μg/ml. Because at this concentration contaminating intact IgG is supposed to be less than 0·02 μg/ml, i.e. a dose that does not induce significant suppression (Tables 1, 3), we conclude that the inhibitory activity of 1E4 is not mediated by ADCC. Thus, the results presented here indicate that 1E4 and HU-4 inhibit both primary and secondary AMLR through a mechanism distinct from an ADCC reaction.

Table 3. Inhibitory effect of 1E4 on secondary AMLR response*

MoAb	Final concentration of MoAb added (μg/ml)	Δc.p.m. ± SEM†	% Inhibition‡
Medium§	—	48,415 ± 4975	0·0
HU-2	2·0	54,221 ± 5777	−12·0
1E4	2·0	779 ± 157	98·4
1E4	0·2	2232 ± 518	95·4
1E4	0·02	59,626 ± 1393	−23·2
1E4	0·002	56,372 ± 1900	−16·4
HU-4	2·0	1513 ± 88	96·9
HU-4	0·2	18,474 ± 4190	61·8
HU-4	0·02	42,488 ± 3254	12·2
HU-4	0·002	51,254 ± 1406	−5·9

* Primary AMLR was cultured for 11 days. Secondary AMLR was established by culturing 5×10^4 AMLR-primed viable cells with 1×10^5 freshly isolated mitomycin C-treated non-T cells in the presence or absence of MoAb. On day 4, the cultures were pulsed with 1 μCi of tritiated thymidine and harvested 8 hr later. T cells alone, 1449 ± 237; non-T cells alone, 1040 ± 357.

†‡ See corresponding footnotes of Table 1.

§ RPMI-1640 supplemented with 10% autologous fresh plasma, 2 mM L-glutamine, 25 mM HEPES, and antibiotics.

Table 4. Inhibitory effect of F(ab')$_2$ fragments prepared from 1E4 on primary AMLR response*

MoAb	Final concentration of MoAb added (μg/ml)	Δc.p.m. ± SEM†	% Inhibition‡
Medium§	—	24,026 ± 3277	0·0
HU-2	10·0	26,287 ± 5586	−9·4
1E4 F(ab')$_2$¶	10·0	1228 ± 390	94·9
1E4 F(ab')$_2$	2·0	820 ± 558	96·6
1E4 F(ab')$_2$	0·2	8127 ± 1658	66·2

* AMLR cultures were established as described in Table 1. T cells alone, 333 ± 147; non-T cells alone, 468 ± 114.

†‡§ See corresponding footnotes of Table 1.

¶ F(ab')$_2$ fragments of 1E4 were generated by pepsin digestion. Contaminating intact IgG was less than 10% as assessed by SDS-PAGE.

DISCUSSION

Recent studies using rabbit antisera to the separated DR α and β subunits have shown that α chain-specific, but not β chain-specific, antisera inhibit T cell proliferative responses in primary and secondary AMLR, suggesting a specific role for the α chain in T cell proliferations in this reaction (Palacios *et al.*, 1982).

The results of the present paper demonstrate that, in contrast to these observations, the two MoAb 1E4 and HU-4, both specific for the β chain of DR antigens, strongly inhibit primary and secondary AMLR through a mechanism distinct from an ADCC reaction, indicating that the inhibition of AMLR is not a unique feature of DR α-specific antibodies and that some DR β-specific MoAbs can eliminate primary and secondary AMLR responses.

Although inhibition of AMLR by chain-specific antibodies does not immediately allow chain localization of an AMLR-stimulating determinant, we infer that the β rather than the α chain of DR antigens is involved in activation of T cells in AMLR by the following reasons. First, AMLR has memory and specificity (Weksler & Kosak, 1977). Second, DR antigens, one of the major target antigens of this reaction, have a mono- or oligomorphic α chain and a highly polymorphic β chain (Kaufman, Andersen & Strominger, 1980; Shackelford & Strominger, 1980; de Kretser *et al.*, 1982), and an allotypic determinant of the DR antigen is located on its β chain (Johnson *et al.*, 1982). Thus, if AMLR indeed represents T cell anti-self Ia reactions, T cells must recognize an antigenic determinant on highly polymorphic β chains in order that this reaction has the classic immunological characteristics of memory and specificity. In this context, the inhibition of AMLR by the two DR β-specific MoAbs shown here appears to provide indirect evidence for the involvement of DR β chains in activation of T cells in AMLR.

It should be emphasized that an assumption of an AMLR-stimulating determinant on DR β chains does not exclude the existence of antibodies that are able to inhibit AMLR but are directed against the α chain or a combinatorial determinant of DR antigens. In fact, xenoantisera specific for the α chain and a MoAb specific for a combinatorial determinant of DR antigens have been reported to inhibit AMLR (Palacios & Möller, 1981; Palacios *et al.*, 1982). Most likely, these antibodies induce steric hindrance of DR antigens to result in covering of an AMLR-stimulating determinant located on the β chain of DR antigens.

In summary, the results presented in this paper demonstrate that the two MoAbs, both specific for the β chain of DR antigens, can inhibit primary and secondary AMLR through a mechanism distinct from an ADCC reaction, suggesting a possible involvement of DR β chains in activation of T cells in AMLR.

ACKNOWLEDGMENTS

The authors are grateful to Ms M. Keira and Ms K. Kaneuchi for their excellent technical assistance. This work was supported in part by the Research Grant for the Intractable Diseases from the Ministry of Health and Wefare of Japan, and by Grants-in-Aid for

Scientific Research (No. 56480122, 58480183) from the Ministry of Education, Science and Culture, Japan.

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