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Immunosuppressive activity of serum from liver-grafted rats: *in vitro* specific inhibition of mixed lymphocyte reactivity by antibodies against class II RT1 alloantigens

M. TSURUFUJI, K. ISHIGURO, T. SHINOMIYA, T. UCHIDA & N. KAMADA* *Mitsubishi-Kasei Institute of Life Sciences, Machida, Tokyo, Japan and *Department of Surgery, University of Cambridge, Addenbrooke's Hospital, Cambridge, U.K.*

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SUMMARY

The immunosuppressive activity of serum from PVG rats following orthotopic transplantation of DA liver has been examined *in vitro*. Liver grafts in this combination are never rejected, but induce a state of specific transplantation tolerance in the recipient. Serum from such tolerant animals was able to inhibit proliferation of normal PVG lymph node cells in response to DA stimulators in the mixed lymphocyte reaction (MLR); inhibition was specific for donor (DA) antigens. Interleukin-2 (IL-2) production during the MLR was also reduced. The production of anti-DA cytotoxic T cells developing in the MLR was not affected, but the total yield of such cells was reduced. Evidence was obtained that part of the inhibitory serum activity was due to IgG antibody against class II $RT1^a$ alloantigens. Thus, a purified IgG fraction retained much of the inhibitory activity which could be removed by an anti-IgG absorbent. Studies of MLR inhibition in different rat strains indicated the anti-class II specificity of the inhibitory IgG. Lymph node cells from DA-liver-grafted PVG rats responded normally against DA stimulators *in vitro*, and this MLR was also blocked by the inhibitory IgG. Our results suggest that anti-class II allo-antibody may play a role in immunosuppression and long-term graft survival following liver transplantation in this combination.

INTRODUCTION

Liver grafting in rats has a variable outcome, which is strictly dependent on the strain combination of donor and recipient used. Thus, in certain combinations, a liver transplant is subject to acute rejection, while in others rejection occurs more slowly or not at all (Limmer, Herbertson & Calne, 1980; Houssin *et al.*, 1980; Engemann *et al.*, 1982; Zimmermann *et al.*, 1983; Kamada, 1985a). We have made a detailed study of one combination, namely DA-liver grafted into PVG recipients, in which the liver graft is never rejected unless the recipients are presensitized against DA (Kamada, Brons & Davies, 1980; Kamada, Davies & Roser, 1981a; Kamada *et al.*, 1983). Normal PVG recipients of such grafts become systemically tolerant of DA antigens, and after liver transplantation will also accept grafts of other DA organs (heart, kidney) and skin (Kamada, Davies & Roser, 1981b; Kamada & Wright, 1984; Kamada, 1985b). The cellular mechanisms behind this tolerant state have been described in previous publications (Kamada *et al.*, 1981b; Davies, Kamada & Roser, 1983; Roser *et al.*, 1983; Kamada & Shinomiya, 1985). There is clear evidence from adoptive transfer experiments for deletion, from the recipient's pool of recirculating lymphocytes, that some of the DA-reactive clones are involved in graft rejection (Davies *et al.*, 1983; Roser *et al.*, 1983; Kamada & Shinomiya, 1985). However, clonal deletion is selective, and the ability to mount anti-DA proliferative responses in the form of mixed lymphocyte and graft-versus-host reactions remains normal in the liver-graft recipients. Thus, at the cellular level, the recipient displays a state of partial or 'split' tolerance (Kamada, 1985b; Kamada *et al.*, 1981b; Davies *et al.*, 1983; Roser *et al.*, 1983; Kamada & Shinomiya, 1985).

It is also possible, therefore, that antibody-mediated mechanisms play a role in tolerance induced by liver grafting. Studies of the antibody response in these rats have demonstrated an early, low level of anti-class I ($RT1A^a$) antibody in serum which declines to background levels within 6 weeks after grafting; in contrast, the antibody response against class II ($RT1B^a/D^a$) allo-

Abbreviations: BSA, bovine serum albumin; CML, cell-mediated lympholysis; Con A, concanavalin A; CTL, cytotoxic T lymphocyte; CTLL, cloned cytotoxic T cell; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; GVHR, graft-versus-host reaction; HPLC, high-performance liquid chromatography; Ig, immunoglobulin; IL-2, interleukin-2; LNC, lymph node cell; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; OLT, orthotopic liver transplantation; PBS, phosphate-buffered saline; TdR, thymidine.

Correspondence: Dr N. Kamada, Dept. of Experimental Surgery, National Children's Medical Research Center, 3-35-31, Taishido, Setagaya-ku, Tokyo 154, Japan.

antigens is strong and maintained at high titres for several weeks post-transplantation (Kamada, 1985b; Zimmermann *et al.*, 1979). In this paper we have examined *in vitro* the immunosuppressive properties of serum from DA-liver-grafted PVG rats. The mixed lymphocyte reaction of lymph node cells both from normal and liver-grafted PVG rats against DA stimulator cells was specifically inhibited by such sera, and evidence was obtained that the inhibition is at least partly due to IgG antibody against class II RT1^a antigens.

MATERIALS AND METHODS

Animals

Rats, 8–16 weeks of age, of the following strains were obtained from Olac (1976) Ltd, Bicester, Oxon, U.K.: PVG (RT1^c), DA (RT1^a), WAG (RT1^u), and the PVG congenic strains PVG.RT1^a and PVG.R1.

Surgical procedures

Orthotopic liver transplantation (OLT) was performed by our previously published techniques (Kamada & Calne, 1983), using male DA rats as donors and male PVG rats as recipients.

Sera

The rat sera used in this study were obtained from PVG recipients of DA-liver grafts killed two to four months after OLT, with normal PVG serum as a control.

Rabbit antiserum against rat IgG was obtained from animals immunized three times, at weekly intervals, with 1 mg rat IgG (Cappel Inc., Cochranville, PA) emulsified in complete Freund's adjuvant, administered into footpads and limbs. Animals were bled 10 days after the final injection.

High-performance liquid chromatography (HPLC)

HPLC was used for gel filtration and ion exchange chromatography. A Tri Rotar-4 system was obtained from Japan Spectroscopic Co. Ltd (Tokyo). Fractionation of rat serum by HPLC gel filtration was carried out using a G3000SW column (Toyo Soda Manufacturing Co. Ltd., Tokyo), dimensions 7·5 mm internal diameter (ID) × 60 cm, at a flow rate of 1·0 ml/min in PBS. Ion exchange chromatography is described below.

Purification of rat IgG from serum

Five mililitres of pooled serum of liver-grafted rats were diluted three times with PBS, and precipitated with ammonium sulphate (40% saturation). The precipitate was dissolved in water and applied to a column of Sephadex G-200 (Pharmacia, Uppsala, Sweden), dimensions 2·4 cm ID × 90 cm, equilibrated with 50 mM Na-phosphate buffer, pH 7·6. Peak IgG-containing fractions were pooled and further purified by HPLC using a prepacked column (7·5 mm ID × 75 mm) of a DEAE ion exchange resin (DEAE-5PW, Toyo Soda Manufacturing Co. Ltd) in 50 mM Na-phosphate buffer, pH 7·6. The non-absorbed protein peak (IgG) was collected and concentrated by ultrafiltration.

Enzyme-linked immunosorbent assay (ELISA)

Rat IgG concentration in serum and purified IgG preparations were estimated in a blocking assay using the ELISA system (Engvall & Perlmann, 1971). Wells of microtitre plates (Nunc Immunoplate-2, Nunc, Roskilde, Denmark) were coated with rat Ig by incubation with 1:300 dilution of normal rat serum in phosphate-buffered saline (PBS) for 1 hr at room temperature. The wells were then washed with PBS and coated again with 2% bovine serum albumin (BSA) in PBS. Standard or unknown samples containing rat IgG were serially diluted in 2% BSA–PBS, mixed with a suitable dilution of rabbit anti-rat IgG specific for rat gamma chain (Cappel Inc.) and incubated for 1 hr at room temperature. One hundred microlitres of each mixture were transferred to Ig-coated wells and incubated for 1 hr, after which the wells were washed and incubated for a further hr with 100 μl of a 1:500 dilution of goat anti-rabbit IgG antibody coupled with horseradish peroxidase (Cappel Inc.). After washing the wells again, 100 μl of 0·1 mg/ml tetramethyl benzidine (Miles Labs Inc.) in 0·1 M acetate buffer pH 6·0 containing 1·3 mM H_2O_2 (Bos *et al.*, 1981) were added to each, and the reaction stopped after 5–10 min by the addition of 50 μl 1 N H_2SO_4. Optical density was read by a microplate photometer (Corona Electronic, Japan) and converted to nanograms of protein by comparison with a standard curve for purified rat IgG or Ig standard rat serum (Miles Labs).

Sepharose-conjugated rabbit anti-rat IgG absorbent

Rabbit anti-rat IgG antibody was purified from serum by affinity chromatography. Rabbit serum was applied to a column of Protein A-Sepharose (Pharmacia). After a wash with PBS, IgG was eluted with 0·1 M glycine-HCl buffer, pH 2·4. IgG-containing fractions were neutralysed with 1 M Tris-HCl, pH 9·0, concentrated by ultrafiltration (PM-30 membrane, Amicon), and coupled to CNBr-activated Sepharose-CL6B (Pharmacia) by the manufacturer's standard procedures. IgG from normal rabbit serum was similarly conjugated as a control. The anti-IgG absorbent was used to remove and isolate immunoglobulins from rat sera, as described in the Results.

Absorption of rat serum with spleen cells

Serum from OLT rats at a 1:100 dilution was incubated with DA, PVG or PVG.R1 spleen cells for 1 hr at room temperature, followed by removal of the cells by centrifugation. Absorption was repeated six times and the absorbed serum then sterilized by millipore filtration.

Radioimmunoassay for rat alloantibodies

Antibodies against class I and class II donor (RT1^a) antigens were detected by a two-stage binding assay using red cells or lymph node cells of the congenic strain PVG.RT1^a and radiolabelled sheep anti-rat immunoglobulin, as described previously (Kamada & Shinomiya, 1986). The rat monoclonal anti-RT1A^a antibody R3/13 (Howard *et al.*, 1980) was used as a positive control and reference standard for both the above assays; R3/13 supernatant was the generous gift of Dr C. Milstein (Laboratory of Molecular Biology, Cambridge, U.K.). The radioimmunoassays using red cells or lymph node cells were of similar sensitivity in detecting R3/13.

Mixed lymphocyte reaction (MLR)

The MLR was performed after the method of Schwartz, Fathman & Sachs (1976) with minor modifications. Lymph node cells, $1–2 \times 10^5$, from either normal PVG rats or PVG liver-graft recipients were incubated with 1, 3·5 or 7×10^5 X-ray irradiated (2000 R) DA or WAG lymph node cells in 200 μl volumes in 96-well flat-bottomed microculture plates (Corning,

New York, NY). The culture medium consisted of RPMI-1640 (Gibco, Grand Island) containing 100 U/ml penicillin, 10 μg/ml streptomycin, 5 μg/ml fungizone, 50 μM 2-mercaptoethanol and 10% FCS (Gibco). After 72 hr at 37° in a humidified atmosphere of 5% CO_2/95% air, the cultures were pulsed with 1 μCi ^{3}H-methyl-thymidine ([^{3}H]TdR), specific activity 6·7 Ci/mmol (New England Nuclear, Boston, MA), and incubated for a further 18 hr. The cells were then harvested onto glass-fibre filter paper strips by an automatic cell harvester and radioactivity counted in a liquid scintillation counter (Beckman). In experiments in which serum from liver-grafted rats or purified IgG from such serum, was added to the cultures, percentage inhibition was calculated as:

$$\frac{\{1-[([^3H]TdR \text{ incorporated in responders with stimulators and sample}) - ([^3H]TdR \text{ incorporation of responders with sample only})]}{[([^3H]TdR \text{ incorporation of responders with stimulators}) - ([^3H]TdR \text{ incorporation of responders alone})]\} \times 100.}$$

Cell-mediated lympholysis (CML)

CML effector cells were induced by mixed lymphocyte culture. PVG lymph node cells, 4×10^5, were incubated with 2×10^5 X-ray irradiated (2000 R) DA lymph node cells in 200 μl volumes as in the MLR described above. Each group was set up in triplicate (12 wells × 3 rows). For inhibition studies, serum from liver-grafted rats was added to the cultures from the outset. After 5 days incubation (conditions as for MLR), cells were recovered, washed in culture medium, counted by trypan blue exclusion and used as effector cells in a ^{51}Cr-release cytotoxicity assay. For the preparation of target cells, $1–2 \times 10^7$ DA lymph node cells stimulated with Con A were labelled by mixing with 200–400 μCi $Na_2{}^{51}CrO_4$, specific activity 250–300 mCi/mg (New England Nuclear), in 200–400 μl RPMI-1640/10% FCS and incubating for 1·5–2 hr. ^{51}Cr-labelled cells were washed three times and finally resuspended at 10^5 cells/ml in RPMI-1640/10% FCS. This cell suspension was dispensed into microculture wells (100 μl/well) and effector cells added to give effector:target ratios of 70:1, 30:1 and 10:1. Plates were incubated for 4 hr, centrifuged at 400 *g* for 5 min, and the radioactivity released determined by counting 100 μl samples from each well in a gamma counter (Beckman).

Assay for Interleukin-2 (IL-2)

IL-2 activity in culture medium after MLR was assayed by incorporation of [^{3}H]TdR into cloned cytotoxic T cells (CTLL) (Gillis *et al.*, 1978). CTLL cells, 5×10^3, in RPMI-1640/10% FCS were seeded into 96-well flat-bottomed microplates (100 μl/well) together with 100 μl MLR supernatant. After 16 hr at 37°, cells were pulsed for 4 hr with 1 μCi[^{3}H]TdR, harvested onto glass-fibre filter strips and counted for radioactivity.

RESULTS

Inhibition of mixed lymphocyte reactivity by serum of liver-grafted rats

Pooled sera from PVG recipients of DA liver grafts (OLT sera) were tested for the ability to affect MLR responses of PVG lymph node cells against DA or WAG stimulators. Table 1 shows evidence of DA-specific inhibition. A significant, but non-specific inhibition of proliferation was produced by normal PVG serum. OLT serum produced a high degree of inhibition (up to 84%) of the PVG anti-DA MLR, but was no more suppressive than normal PVG serum in the anti-WAG MLR; in fact, it tended to enhance this reaction, for reasons unknown.

Effect of OLT serum on cell-mediated lympholysis

OLT serum did not appear to interfere with the proportion of cytotoxic T cells developing in the MLR. Thus, as Table 2 shows, CML activity showed no sign of inhibition at any of the effector:target cell ratios tested. However, there was a significant reduction in the yield of cells from CML cultures, reflecting the inhibition of proliferation noted above.

Immunoglobulin nature of inhibitory material in OLT serum

Three experiments established that inhibition of MLR by OLT serum was mainly due to immunoglobulin.

(a) Fractionation of OLT serum by HPLC gel filtration yielded two major activity peaks (retention times 11–13 and 15–18 min, respectively, Fig. 1a). The second peak contained IgG,

Table 1. Inhibition of mixed lymphocyte reaction by OLT rat sera

Stimulator	*In vitro* addition*	[^{3}H]TdR-incorporated (c.p.m. ± SD) no. of stimulator cells × 10^{-5} 0	1	3·5
DA	—	2700 ± 280	10,800 ± 840	26,000 ± 3700
	Normal PVG serum	500 ± 50	5700 ± 200 (35·8%)	16,300 ± 1400 (32·2%)
	OLT PVG serum-1	200 ± 30	1500 ± 200 (84·0%)	6900 ± 800 (71·2%)
	OLT PVG serum-2	600 ± 190	2700 ± 730 (74·1%)	8800 ± 330 (64·8%)
WAG	—	2500 ± 100	11,200 ± 840	23,600 ± 1000
	Normal PVG serum	660 ± 20	4800 ± 1100 (52·4%)	17,900 ± 600 (18·3%)
	OLT PVG serum-1	300 ± 20	4300 ± 370 (54·0%)	21,600 ± 1800 (0%)
	OLT PVG serum-2	400 ± 70	7100 ± 1200 (22·9%)	26,300 ± 3600 (−22·7%)

OLT, orthotopic liver transplantation.
PVG, lymph node cells were used as responders.
Percentage inhibition in parentheses, calculated as described in the Materials and Methods.
* Serum was added at the beginning of the culture; final concentration 2·5% per well.

Table 2. Effect of OLT rat sera on the induction of cytotoxic T lymphocytes in mixed lymphocyte cultures

In vitro addition*	Cellular response† (cell no. ± SD) × 10^{-6}	% specific ^{51}Cr release at effector:target ratio 70:1	30:1	10:1
—	9·7 ± 1·01	78	74	61
Normal PVG serum	15·2 ± 0·88	73	61	52
OLT PVG serum-1	7·7 ± 0·46	69	62	43
OLT PVG serum-2	7·6 ± 0·19	71	74	55
OLT PVG serum-3	5·9 ± 0·55	74	79	69

PVG lymph node cells were used as responders.

* Serum was added at the beginning of the culture, final concentration 2·5% per well.

† Yield of cells from mixed lymphocyte cultures for preparation of effectors after 5 days' incubation.

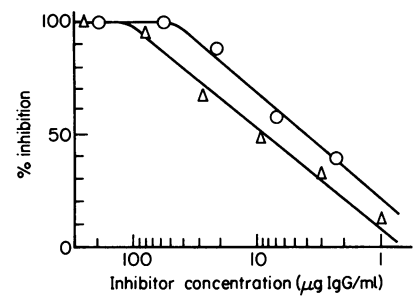

Figure 2. Inhibition of MLR by IgG purified from OLT serum. In the MLR, 2×10^5 normal PVG lymph node cells as responders were mixed with 7×10^5 X-ray irradiated DA lymph node cells. Dilutions of OLT rat serum (O——O) or purified IgG (Δ——Δ) were added at the beginning of the culture to give the final concentration in the culture as shown. Percentage inhibition of MLR was calculated as described in the Materials and Methods.

while the first had molecules of larger size or aggregates. MLR-inhibitory activity was associated with both peaks, as shown.

(b) Ig was specifically removed from OLT serum by passage over a polyvalent rabbit anti-rat Ig absorbent. This led to removal of 99% of the rat-serum Ig, and an almost complete disappearance of MLR-inhibitory activity, compared with OLT serum passed over a column of normal rabbit IgG. Adsorbed Ig was eluted from the column with 0·1 M glycine buffer, pH 2·4, and analysed on a column of G3000SW by HPLC gel filtration. As shown in Fig. 1b, two major protein peaks were identified as IgM and IgG from their retention times and antigenic properties. MLR-inhibitory activity was mainly associated with the second peak containing IgG.

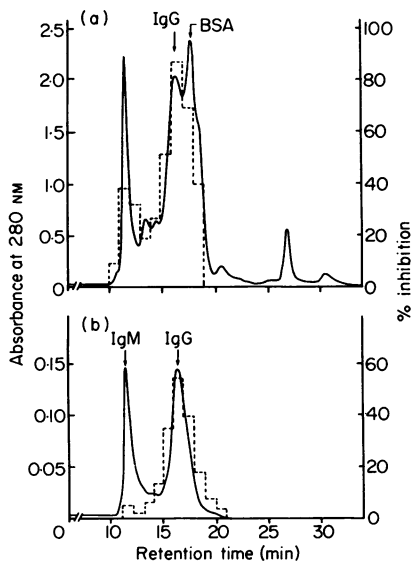

Figure 1. Fractionation of MLR inhibitory activity in OLT serum by HPLC gel filtration. (a) 0·2 ml of OLT PVG serum and (b) 0·2 ml of Ig fraction purified by the anti-rat Ig adsorbent were passed through a G3000 SW column respectively, as described in the Materials and Methods, at a flow rate of 1·0 ml/min. Fractions were collected at 1 min intervals and tested for inhibitory activity in MLR. IgM, IgG and BSA (arrowed) indicate the retention times of standard proteins. (———), absorbance at 280 nm; (– – – –), MLR inhibitory activity.

Table 3. DA-stimulator cells as the target for OLT serum in inhibition of the mixed lymphocyte reaction

Responder	Treatment of responder cells*	Treatment of stimulator cells†	[^{3}H]TdR incorporation c.p.m. ± SD
PVG	—	—	110,000 ± 16,700
PVG	—	PVG IgG	152,000 ± 25,900
PVG	—	OLT IgG	47,000 ± 7100
PVG	PVG IgG	—	104,000 ± 11,200
PVG	OLT IgG	—	101,000 ± 8900
PVG	OLT IgG	OLT IgG	39,500 ± 6600
WAG	—	—	134,000 ± 16,100
WAG	—	PVG IgG	157,000 ± 16,900
WAG	—	OLT IgG	99,800 ± 10,300
WAG	PVG IgG	—	141,000 ± 11,500
WAG	OLT IgG	—	242,000 ± 12,400
WAG	OLT IgG	OLT IgG	151,000 ± 15,400

Concentrations of responder and stimulator cells were 2×10^5 and $3·5 \times 10^5$ cells/well, respectively.

* 2×10^7 normal PVG or WAG lymph node cells treated with 4 ml IgG (0·1 mg/ml) in the culture medium for 2 hr at 0°.

† 2×10^7 normal DA lymph node cells treated as in the above case.

(c) IgG purified from OLT serum by gel filtration on Sephadex G-200 followed by DEAE-5PW chromatography was also able to suppress the MLR. The purity of the IgG isolated in this way was estimated to be above 96% by SDS-gel electrophoresis and Coomassie brilliant blue staining (Laemmli, 1970). The MLR-inhibitory activity was completely associated with the IgG protein peak by HPLC analysis on a column of G3000SW (data not shown). The titration curves for inhibition of MLR by OLT serum and purified IgG were parallel (Fig. 2). Taking into account the relative concentrations of IgG in the serum (22·3 mg/ml) and purified preparation (3·2 mg/ml), it appears that the isolated IgG has about half of the inhibitory

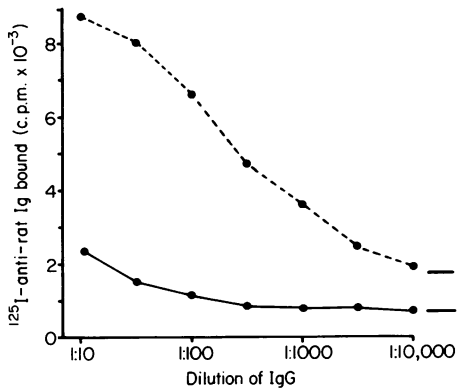

Figure 3. Titration of antibodies against class I and class II RT1^a antigens in IgG purified from OLT PVG serum. IgG was assayed against PVG.RT1^a red cells (———) for anti-class I activity, and PVG.RT1^a lymph node cells (– – – –) for anti-class II, with detection by ^{125}I-labelled sheep anti-rat Ig. The background of each assay is indicated by a bar. (Higher background with lymph node cells is due to binding of the labelled anti-Ig reagent to rat B cells).

activity per mg of the OLT serum. The extra effectiveness of serum may be due to the additional non-specific inhibition already seen in Table 1.

Target cell of MLR inhibition

To determine the cellular target of action of the inhibitory IgG in OLT serum, responder (PVG) or stimulator (DA or WAG) lymph node cells were treated separately with the purified IgG before setting up the MLR. Table 3 shows that treating PVG responder cells had no effect on their subsequent proliferative responses, whereas pretreatment of DA stimulator cells led to marked inhibition. Reactivity of third party cells (WAG) was inexplicably enhanced by preincubation in OLT IgG.

Antigenic specificity of inhibitory IgG

The presence of allo-antibody activity against class I and class II DA (RT1^a) antigens in the IgG purified from OLT serum was determined by two-stage binding radioimmunoassay against PVG.RT1^a red cells or lymph node cells. The titration curves

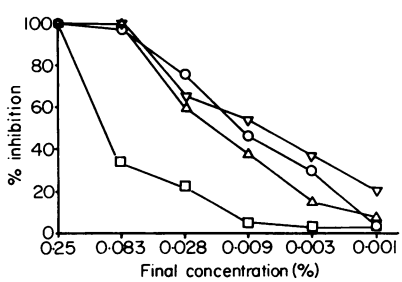

Figure 4. Inhibition of MLR by OLT rat serum after absorption with rat spleen cells. In the MLR, 2×10^5 normal PVG lymph node cells as responders were mixed with $3{\cdot}5 \times 10^5$ X-ray irradiated DA lymph node cells. OLT serum either unabsorbed (○——○) or after absorption with DA (□——□), PVG (△——△) or PVG.R1 (▽——▽) spleen cells was titrated and added at the beginning of the cultures to give the final dilution as shown.

showed a low anti-class I (RT1A^a) titre, with an end-point of about 1:30 dilution, but a much higher titre of anti-class II (TR1B^a/D^a) antibody, with an end-point of about 1:1000 (Fig. 3). Since the sensitivities of the red cell and lymph node cell binding assays for a monoclonal anti-RT1A antibody (R3/13) were approximately equal, the anti-class II level can be said to be at least 30 times higher than the anti-class I in this IgG preparation. Comparison with a standard curve constructed using the R3/13 monoclonal suggested that the concentration of anti-class I IgG was 1–2 μg/ml, while that of anti-class II was 50–100 μg/ml (total IgG was 3·2 mg/ml).

To further establish that the inhibitory activity was due to anti-class II specific antibody, two experiments were carried out in the MLR. OLT IgG was absorbed three times with DA, PVG.R1 or PVG spleen cells, and inhibitory activity re-assayed. Figure 4 shows that DA spleen cells removed about 90% of the inhibitory activity, while PVG.R1 and PVG cells were ineffectual absorbers. In addition, OLT IgG was added to a congenic recombinant RT1 combination in the MLR. Table 4 shows that OLT IgG inhibited PVG.R1 anti-PVG.RT1^a MLR (Exp. 2). Since PVG.R1 cells carry the class I antigens of DA (RT1A^a) with the class II antigens of PVG (RT1B^c/D^c) (Butcher & Howard, 1977), these results imply that inhibition of MLR is due to anti-class II antibodies, which would be removed by absorption with the DA class II antigen.

Inhibition of MLR reactivity of lymph node cells from liver-grafted rats

It has been shown previously that the anti-DA MLR potential of lymph node cells from PVG recipients of DA liver transplants is normal, despite the fact that such rats are systemically tolerant of DA (Kamada, 1985b; Kamada *et al.*, 1981b; Davies *et al.*, 1983; Roser *et al.*, 1983, Kamada & Shinomiya, 1985). It was therefore of particular interest to see whether an MLR mounted by such cells was inhibitable by OLT serum from similar, DA-tolerant rats, and the purified IgG fraction of OLT serum, both of which suppressed the anti-DA MLR of normal PVG lymphocytes. Table 4 (Exp. 3) shows that this is indeed the case, and that inhibition was again DA specific.

Inhibition of IL-2 production by OLT serum and purified IgG

The level of IL-2 was examined in supernatants of mixed lymphocyte cultures in the presence or absence of OLT serum or the purified IgG fraction. In this experiment, the MLR was set up with lymph node cells from liver-grafted rats as responders (see above), and irradiated DA lymph node cells as stimulators. Figure 5 shows that OLT serum suppressed IL-2 production throughout the 3-day period of observation, while IgG was rather less effective during the early part of the culture, but caused complete inhibition by Day 3.

DISCUSSION

The mechanisms behind transplantation tolerance induced by liver grafting in rats are complex (Kamada, 1985a). They include the deletion of alloreactive T cells from the recirculating pool, as demonstrated by adoptive transfer experiments (Davies *et al.*, 1983; Roser *et al.*, 1983; Kamada & Shinomiya, 1985) and, as shown in this and a previous report (Kamada *et al.*, 1986), the

Table 4. Mixed lymphocyte reaction of lymph node cells from various rats; inhibition by OLT serum or purified IgG

No. of exp.	Responder	Stimulator	*In vitro* addition* (mg IgG/ml)	[^{3}H]TdR incorporation† c.p.m. ± SD
1	PVG	—	—	3700 ± 500
	PVG	PVG.RT1^a	—	138,000 ± 10,700
	PVG	PVG.RT1^a	OLT IgG (0·1)	67,200 ± 5600
	PVG	PVG.RT1^a	OLT IgG (0·01)	102,000 ± 17,200
	PVG	PVG.RT1^a	normal IgG (0·1)	143,000 ± 11,200
2	PVG.R1	—	—	7900 ± 2100
	PVG.R1	PVG.RT1^a	—	40,600 ± 9500
	PVG.R1	PVG.RT1^a	OLT IgG (0·1)	21,600 ± 6100
	PVG.R1	PVG.RT1^a	OLT IgG (0·01)	23,900 ± 4800
	PVG.R1	PVG.RT1^a	normal IgG (0·1)	42,700 ± 9900
3	OLT PVG‡	—	—	8200 ± 1300
	OLT PVG	DA	—	95,200 ± 3800
	OLT PVG	DA	OLT IgG (0·1)	51,500 ± 5800
	OLT PVG	DA	OLT serum (0·6)	10,100 ± 1600
	OLT PVG	WAG	—	111,000 ± 6500
	OLT PVG	WAG	OLT IgG (0·1)	178,000 ± 11,200
	OLT PVG	WAG	OLT serum (0·6)	168,000 ± 6600

* Purified IgG or serum were added at the beginning of the culture. The IgG concentration of OLT serum was estimated by ELISA.

† Number of responder and stimulator cells were 2×10^5 and $3{\cdot}5 \times 10^5$ cells/well respectively.

‡ Responder cells from PVG rats grafted with DA-liver 12 weeks previously.

presence of suppressive molecules in serum. Among the latter, allo-antibodies, in particular those with class II antigen specificity, seem to be important suppressive agents. The inhibition of MLR by antisera against class II antigens on stimulator cells has been well established (Schwartz *et al.*, 1976; Meo *et al.*, 1975). Here, we have shown that the proliferation of normal PVG lymph node cells in an MLR against DA stimulators can be specifically blocked by serum from PVG rats tolerized against DA by liver grafting and by the IgG fraction purified from such sera. Absorption studies make it very likely that MLR-inhibition is due to class II antibodies reactive with the DA stimulator cells; the latter, rather than the PVG responder cells, were shown to be the targets of inhibition of the MLR. This tends to rule out a role for anti-idiotypic antibody in the inhibition, and suggests that masking of class II antigens on stimulator cells is the mechanism. We have also confirmed the previous observation that lymph node cells from PVG recipients of DA-liver grafts are able to mount normal MLR responses against DA antigens, despite the existence in the recipient of systemic transplantation tolerance against DA (Kamada *et al.*, 1981b; Davies *et al.*, 1983; Roser *et al.*, 1983); this MLR can also be specifically inhibited by serum and IgG from similarly tolerant rats. We therefore conclude that antibody against class II donor antigens may be one means of long-term suppression of specific cell-mediated immune reactivity in liver-graft recipients.

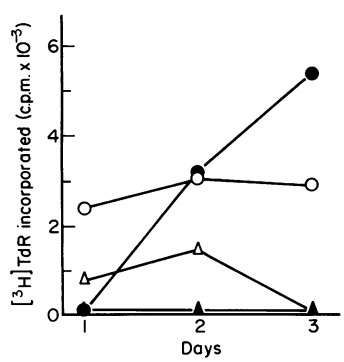

Figure 5. Inhibition of production of IL-2 in MLR supernatants by OLT rat serum or its purified IgG. MLR culture conditions as in Fig. 3, with addition of normal PVG serum (●——●), OLT rat serum (▲——▲) or its purified IgG fraction (△——△) from the outset at a final concentration of 0·5 mg IgG per well. (○——○), control without serum addition. IL-2 activity in MLR supernatants was measured on Days 1, 2 or 3. Background incorporation into CTLL cells in medium alone was less than 200 c.p.m.

These results help to clarify a paradoxical situation revealed when events within the liver graft are compared with those in the whole animal. Thus, there is clear histological evidence that PVG recipients mount a rejection response during the first 2 weeks after DA-liver grafting, with mononuclear cell infiltration of portal tracts and sinusoids (Kamada *et al.*, 1983). However, by 4 weeks, the cellular invasion has virtually disappeared, and at subsequent times the appearance of the liver graft is essentially normal. Class I reactive T cells seem to be physically eliminated at this stage. The paradox lies in the finding that lymph node cells from long-term surviving tolerant rats have an apparently normal ability to mount proliferative cell-mediated alloresponses against class II antigens, both *in vivo*, when transferred into secondary hosts (graft-versus-host reaction) (Kamada *et al.*, 1981b; Davies *et al.*, 1983; Roser *et al.*, 1983; Kamada & Shinomiya, 1985), and *in vitro*, when stimulated by DA in the MLR (Kamada *et al.*, 1981b; Davies *et al.*, 1983;

Roser *et al.*, 1983; and this paper), a situation which we have described as split tolerance (Kamada, 1985a). Thus, it was clear that some additional factors must act to prevent anti-class II responses occurring within the liver graft after about the 4th week post-transplantation. Antibody, antigen and immune complexes have all been considered as possible specific, soluble suppressive agents (Roser *et al.*, 1983). The antibody response has been investigated in detail (Kamada, 1985b; Zimmermann *et al.*, 1979; Kamada & Shinomiya, 1986). The class I antibody response to DA RT1 antigens in PVG following a liver graft is weak, and after 3 weeks shows clear signs of tolerance induction; this is partly accounted for by the inherited low responsiveness of PVG against DA (Butcher *et al.*, 1982; Howard & Butcher, 1981). In contrast, the anti-class II antibody levels reach a high titre within 3–4 weeks after DA-liver grafting, and only show signs of declining in the fourth month (Kamada, 1985a; Zimmermann *et al.*, 1979; Kamada & Shinomiya, 1986). Our present observations suggest that these antibodies are at least partly responsible for the absence of proliferative T-cell reactions within the liver graft after 4 weeks, and by implication also for suppressing rejection reactions dependent on class II antigen recognition, release of lymphokines and chemotactic influx of macrophages. On the other hand, our quantitative comparison indicated that purified IgG did not seem to contain all the inhibitory activity of the original serum. Some of the latter may have been due to free alloantigen, which has been demonstrated in OLT serum (Kamada *et al.*, 1980; Kamada *et al.*, 1981b; Roser *et al.*, 1983; Zimmermann *et al.*, 1979), or complexes (Kamada *et al.*, 1981b; Roser *et al.*, 1983). The second possibility might explain why inhibitory activity was found in the exclusion peak when OLT serum was fractionated by gel filtration column (Fig. 1).

We have also shown that, in addition to suppression of proliferation, production of at least one major T-cell product, IL-2, is inhibited by OLT serum and its purified IgG during the MLR, and this may help to prevent development of cytotoxic T cells *in vivo*. Potentially, therefore, anti-class II antibodies induced by liver grafting can be expected to be potent immunosuppressive agents. Their effectiveness *in vivo* has indeed been demonstrated by the passive enhancement of semi-allogenic (DA × PVG)F_1 heart grafts in PVG recipients (Kamada, 1985a; Roser *et al.*, 1983), and more recently by enhancement of fully allogeneic heart grafts (PVG.RT1^a into PVG) (Kamada *et al.*, 1986).

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