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Examination of Serum Class I Antigen in Allograft Recipient Rats –Origin and control of serum class I antigen–

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ABSTRACT

We examined the appearance of DA type ($RT1A^a$) class I antigen in the serum of rats that had received isogeneic or allogeneic liver grafts (DA into DA, DA into LEW, PVG into DA, PVG into F1 hybrid (DAxPVG). Recipient LEW rats were given either one injection of the anti-CD8 mAb, OX-8, following thymectomy or anti-CD4 mAb (cocktail of OX-35 and OX-38) following thymectomy 3 days prior to liver grafting. We also tested the serum $RT1A^a$ antigen titer of F1 (DAxPVG) recipients after PVG spleen transplantation and the serum $RT1A^a$ antigen titer in the DA rat after hepatectomy and cyclosporin treatment.

Replacement of DA liver by PVG lowered transiently the serum level of $RT1A^a$ antigen to 70% of that in normal DA serum, shortly after liver transplantation. However, this titer increased gradually over the level in normal DA serum.

A PVG spleen graft to an F1 hybrid recipient resulted in death due to typical GVH disease between 13 and 24 days after spleen transplantation. The $RT1A^a$ antigen titer increased to several times more than that in normal F1 serum throughout the observation period.

LEW recipients of DA liver died at 9–11 days (9.8 ± 1.1 days) due to acute liver rejection. In this combination, the serum level of $RT1A^a$ increased until day 8, reaching a maximum (four times) on day 4. Deletion of either $CD8^+$ or $CD4^+$ T cells by anti-CD8 or anti-CD4 MAb in this transplantation prolonged the survival times of liver grafts for up to 26.8 ± 8.4 and 35.6 ± 17.9 days, respectively. In the anti-CD8 or anti-CD4 MAb- treated recipients, the serum titer of DA class I antigen was not elevated and there were no differences between the two. Hepatectomy in combination with cyclosporin induced a high titer of liver- derived class I antigen in the serum as long as liver regeneration proceeded.

These results suggest that the liver is the principal source of serum class I antigen in rats. Rejection, GVH reaction and liver regeneration increased the serum class I antigen from transplanted liver or host tissue. It is unlikely that this is due to cleavage of membranous class I antigen by class I- reactive $CD8^+$ T cells.

Key words: *Serum class I antigen, Rat liver transplantation, $CD8^+$ T cells $CD4^+$ T cells*

It has been demonstrated that humans, rats and mice have class I antigen in their serum[1,3,18,24]. However, its origin and physiological role has not been fully determined. It has been reported that donor liver- derived class I antigen appears in the circulation of liver allograft recipients after transplantation under experimental conditions and clinics[10,17].

Kamada et al[11,25] have shown that soluble serum class I antigen secreted from the liver graft block CTL receptors from target cell lysis and is responsible for preventing liver rejection. On the other hand, Davies et al[2] and Pollard et al[16] have shown that the serum level of class I antigen in human liver graft recipients parallels the intensity of liver rejection. Thus, monitoring of the serum antigen

Abbreviations: GVH, graft-versus-host; CTL, cytotoxic T lymphocyte; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; MAb, monoclonal antibody; HVG, host-versus-graft

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titer becomes a good indicator of liver rejection. Thus, controversy exists over the physiological role of donor liver- derived class I antigen after liver trasplantation and there is no consensus as to whether the appearance of serum class I antigen is the result of liver rejection or tolerance[5,14].

In the present experiment, using various transplant models in rats, we examined the role and control of serum class I antigen after liver transplantation or liver regeneration.

MATERIALS AND METHODS

Animals

Rats of the following strain were obtained from Clea, Tokyo, Japan: PVG (MHC haplotype $RT1^c$), DA($RT1^a$), LEW($RT1^l$), and F1 hybrid (DAxPVG) $RT1^{a/c}$.

Materials

Chemicals were purchased from Sigma (St Louis, MO) except where otherwise noted. Anti-mouse immunoglobulin, β -galactosidase-linked antibody (from sheep IgG) was a product of Amersham International (cat. no. NA. 831: Amersham, UK). An enzyme substrate, chlorophenol red- β -D galactopyranoside sodium salt (CPRG), was purchased from Boehringer-Mannheim (Mannheim, Germany).

Antibodies

A monoclonal antibody (MAb), OX-18, a mouse IgG1 antibody recognizing a non-polymorphic determinant of rat MHC class I antigen, was purchased from Serotec (Oxford, UK). A monoclonal antibody, R3/13[8], a rat IgG2b antibody against $RT1A^a$ antigen, was kindly provided by Dr C. Milstein.

The monoclonal antibodies used in the in-vivo study were OX-35 and OX-38 for anti-CD4, and OX-8 for anti-CD8 α chain. These MAbs were generously provided by A.F. Williams and D.W. Mason (Oxford).

Enzymic immunoassay for serum class I antigen (Fig. 1)

The wells of plastic microtiter plates (Nunc Immunoplate II, Roskilde, Denmark) were coated with an immunoglobulin fraction of monoclonal antibody R3/13 (Howard et al, 1980) by incubating overnight 50μl of the antibody at 50μg immunoglobulin/ml in phosphate-buffered saline (PBS). The antibody was removed and the remaining protein binding sites were blocked by adding to each well 300μl of dilution buffer (PBS containing 0.1% bovine serum albumin. 0.05% Tween 20 and 0.05% NaN_3) for 30 min. The wells were then washed and 100μl of two-fold serial dilutions of the various samples in the dilution buffer were added to the plate for 1 hr. The wells were washed with PBS and 100μl of OX-18 diluted 1/200 was added for 1hr. The wells were washed with PBS and then 100μl of B-galactosidase-linked anti-mouse immunoglobulin diluted 1/200 were added for 1hr. The wells were washed, followed by addition of 100μl of 0.2mg/ml CPRG in PBS containing 1mg /ml BSA, 0.01% 2-mercaptoethanol, 2mM $MgCl_2$ and 0.05% NaN_3, and incubated for 60 min at room temperature. Activities were measured by a Multiscan MC microplate reader (Flow Laboratories, McLean, VA) using filters at 540nm and 690nm. DA serum samples were pooled from 10 female and 10male rats, and used as a standard for DA soluble class I antigen measurement.

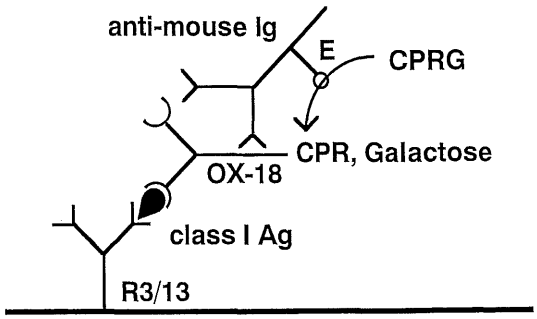

Fig. 1. Schematic representation of enzyme immunoassay for serum class I antigen.
R3/13: Anti-class I ($RT1A^a$) monoclonal antibody.
OX-18: Anti-rat class I MHC monoclonal antibody.
E: β-galactosidase. CPRG: Chlorophenol red-β-D galactopyranoside sodium salt.

Surgical procedures

The methods used for hepatectomy and orthotopic liver transplantation in rats have been described previously[12]. Orthotopic liver transplantation was performed from DA into DA, DA into PVG, PVG into DA, and PVG into F1(PVGxDA). LEW rats with either anti-CD8 MAb (OX-8: 0.4 ml of ascites form) administration plus thymectomy or anti-CD4 MAb (0.8ml of cocktail of MAb W3/25 and OX-38; 0.4 ml each) administration plus thymectomy before liver transplantation. Adult thymectomy of the recipient LEW rats was performed 2 weeks prior to liver transplantation according to standard procedures[7]. The method of heterotopic spleen grafting was described previously and PVG spleen grafting was performed in the right neck of F1 recipient rats[22]. 70% hepatectomy was performed in normal DA rats according to the method of Higgins & Anderson[6]. Cyclosporin was dissolved in olive oil and given intramuscularly to rats at a dose of 20-30 mg/kg per day for 14 consecutive days.

Blood samples were collected sequentially from a tail vein after the operation. Sera were obtained after centrifugation at 2000G for 1 min. Survival times were recorded and compared among groups. An autopsy was performed on every rat to deter-

Table 1. Survival data

Graft	Strain combination (RT1-A)	Treatment	Survival days(n)
Liver	DA(a) ⟶ DA(a)		>100×(10)
	DA(a) ⟶ PVG(c)		>100×(10)
	DA(a) ⟶ LEW(1)		9.8±1.1(10)
	PVG(c) ⟶ DA(a)		>100×(3)
	PVG(c) ⟶ F1(DA×PVG)(a/c)		>100×(6)
	DA(a) ⟶ LEW(1)	Thymectomy+ Anti-CD8α mAb	26.8±8.4(5)
	DA(a) ⟶ LEW(1)	Thymectomy+ Anti-CD8α mAb	35.6±17.9(6)
Spleen	PVG(c) ⟶ F1(DA×PVG)(a/c)		17.8±3.5(5)

mine the cause of death and every liver specimen was subjected to histological study.

RESULTS

Survival

The survival times (days) of the rats which underwent liver transplantation (DA into DA, DA into PVG, PVG into DA, PVG into F1 (PVGxDA), and DA into LEW) with or without one shot injection of anti-CD8 MAb following thymectomy, or anti-CD4 MAb injection following thymectomy, are shown in Table 1. The survival of F1 recipient rats carrying a PVG spleen graft are also shown. Hepatectomized rats, with or without cyclosporin administration survived throughout the observation period in every case. In the combination of DA into PVG or PVG into DA, which are known non-rejection combinations, recipient rats survived indefinitely without any immunosuppressant.

In contrast, with the combination of DA into LEW, a rejection combination, the survival time of untreated LEW rats was 9.8± 1.1 days and all rats died of acute rejection. Anti-CD8 monoclonal antibody treatment of thymectomized LEW rats prior to liver transplantation depleted $CD8^+$ T cells from 15.2% to 0.2%. In contrast, administration of anti-CD4 monoclonal antibody depleted $CD4^+$ T cells from 58.7% to 24.4% (Data not shown). The survival times of DA liver grafts in thymectomized LEW recipients with either anti-CD8 MAb or anti-CD4 MAb treatment were 26.8±8.4 days or 35.6± 17.9 days, respectively, and survival was prolonged in rats treated with anti-CD4 mAb compared to those treated with anti CD8 mAb, although cell depletion was greater in rats with anti-CD8 MAb. With the combination of parental rat graft into F1 hybrid recipient rats, where rejection (HVG) does not occur, but graft-versus-host (GVH) does, the F1 recipient rat of a PVG liver survived for a long period without GVH disease. However, a F1 rat with a spleen graft died within 23 days due to a typical GVH reaction. Indeed, all rats lost weight and hair, developed red paws and suffered from diarrhea until their death.

Serum class I antigen

At least three rats were studied for each experimental condition, in which serum class I ($RT1A^a$) antigen titers were measured. The assay for class I is a two-site sandwich enzyme immunoassay of two-fold serial dilutions of serum with pooled DA rat serum as the standard control or test serum (Fig.1). To compare the levels of antigen in the resultant S-shaped curves, the horizontal displacements of the central near-linear portions of these curves were measured.

Figures 2 and 3 show the class1 antigen levels in serum of isogeneic and allogeneic liver grafted rats. In the transplantation of DA into DA, (isogeneic combination), the serum titer of $RT1A^a$ antigen changed minimally before and after liver grafting throughout the observation period (Fig. 2a). When DA liver was replaced by PVG liver, the $RT1A^a$ antigen titer decreased to about 70% of that in normal DA serum shortly after liver transplantation. However, its titer increased gradually to several times that in normal DA serum (Fig.2b). This suggests first that the liver is the principal source of serum class I antigen and second that the host-versus-graft (HVG) reaction by lymphocytes causes an increase in the recipient's serum class I antigen, presumably due to secretion of $RT1A^a$ antigen from proliferating lymphocytes. This hypothesis is also confirmed by experiments using a parental into F1 hybrid liver transplantation combination, where rejection does not occur (Fig.2c).

Normal F1 rats contain approximately 30–40% $RT1A^a$ antigen of those levels seen in normal DA serum. When F1 liver was replaced by parental PVG liver, the serum titer of $RT1A^a$ decreased transiently shortly after liver grafting, as in the case of PVG into DA liver transplantation described above. However, the serum $RT1A^a$ titer did not increase throughout the observation period.

Data for the rejection combination of DA into

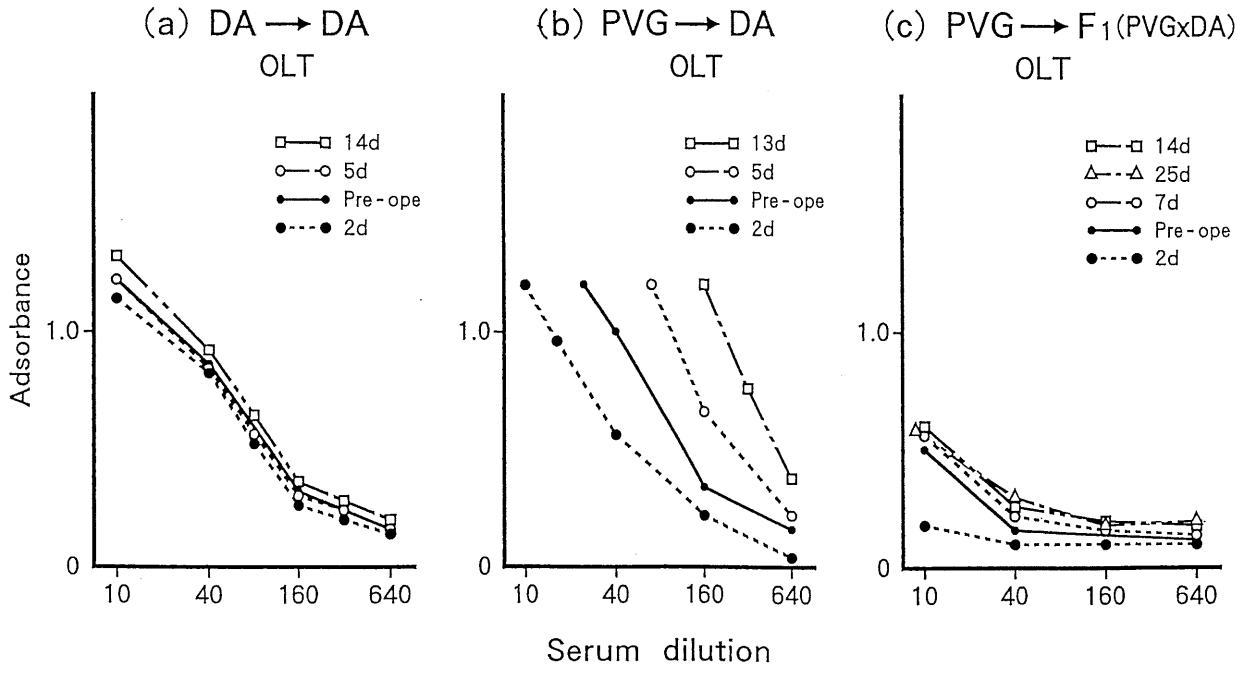

Fig. 2. Serum class I antigen in liver-transplanted rats.
(a) DA rat given an isogeneic liver graft. (b) DA rat given a PVG liver transplant. (c) F1 hybrid rat given a PVG liver graft. Sera were taken at various days after operation. Solid lines in the figure show titration curves of class I antigen in the serum of rats prior to operation.

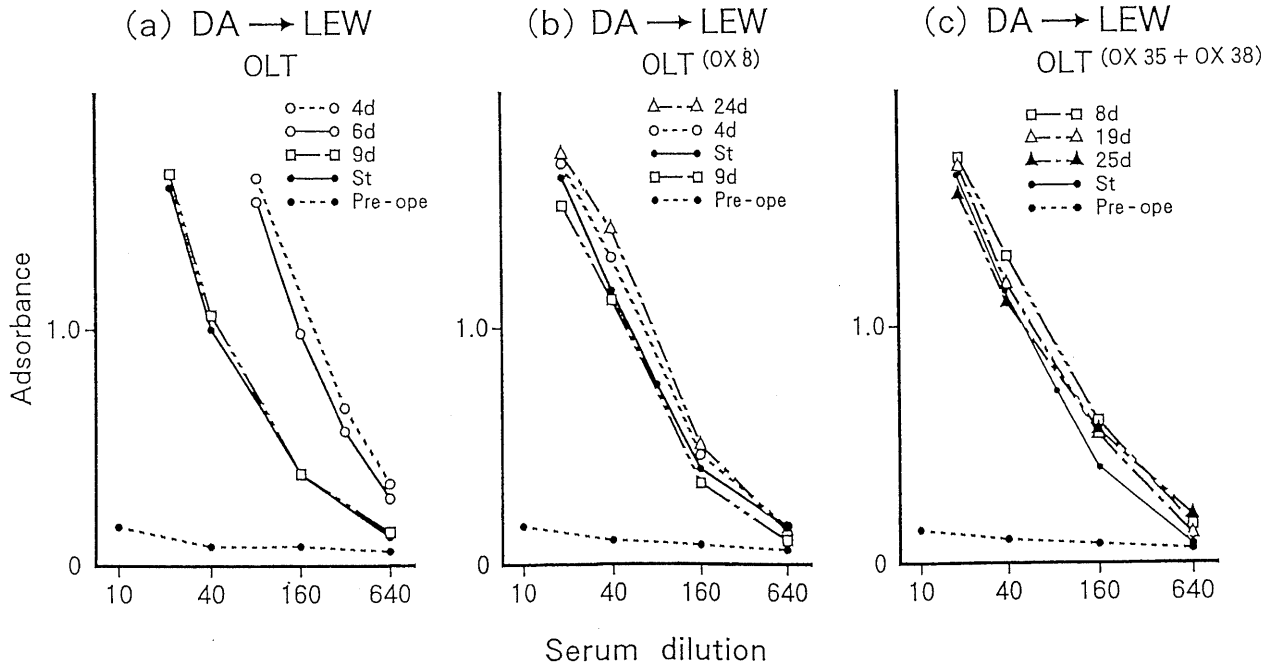

Fig. 3. Serum class I antigen in liver- transplanted rats.
(a) Untreated LEW rat given a DA liver graft. (b) Thymectomized LEW rat (anti-CD8 mAb administration) given a DA liver graft. (c) Thymectomized LEW rat (anti-CD4 mAb administration) given a DA liver graft. Sera were taken at various days after operation. Solid lines in the figure show titration curves of serum class I antigen in DA standard serum.

Table 2. Serum class I antigen in rats with or without cyclosporin treatment and hepatectomy

	Days after treatment					
	1d	2d	3d	7d	14d	28d
(a) Cyclosporin alone	0.95±0.11,	0.93±0.10,	0.66±0.12,	1.04±0.08,	1.17±0.10,	0.92±0.13
(b) Hepatectomy alone	2.72±0.14,	1.59±0.21,	1.67±0.23,	0.96±0.17,	1.01±0.13,	1.01±0.11
(c) Hepatectomy plus cyclosporin	2.00±0.33,	2.50±0.25,	2.31±0.32,	1.71±0.23,	1.95±0.68,	1.19±0.33

Amount of serum class I antigen was assayed by two-site enzyme immunoassay. Relative concentrations were expressed as a ratio comparing the titration curves of cyclosporin A-treated DA rat serum (a), 70% hepatectomized DA rat serum (b) or 70% hepatectomized DA rat serum in combination with cyclosporin A treatment (c) with the standard curive of normal DA serum. The calculated rations of concentrations were expressed as mean±SD.

LEW is shown in Fig.3a. In this case, the level of serum class I antigen increased to a high level by day 4 (four times), and continued to increase until the 8th day and the animals died eventually due to liver rejection. When $CD8^+$T cells are deleted from the LEW rat prior to liver grafting, the serum antigen level does not increase until death (Fig.3b). Similarly, this titer also did not increase when only $CD4^+$T cells are deleted from the recipient rat (Fig.3c). These results suggest that $CD8^+$T cells may not play a principal role in the appearance of liver- derived class I antigen.

The change in serum titer of class I antigen in rats treated with either cyclosporin alone, hepatectomy alone or cyclosporin plus hepatectomy is shown in Table 2. Cyclosporin treatment of the normal DA rat lowered slightly the serum class I $RT1A^a$ antigen titer during the early period of treatment. However, it returned to its former level by 7 days. In the case of hepatectomized normal DA rats, the serum antigen increased markedly on the first day after liver resection, but decreased gradually to the normal level by 7 days. In contrast, cyclosporin treatment in combination with hepatectomy increased the titer to more than that in rats which underwent hepatectomy alone during at least 2 weeks .

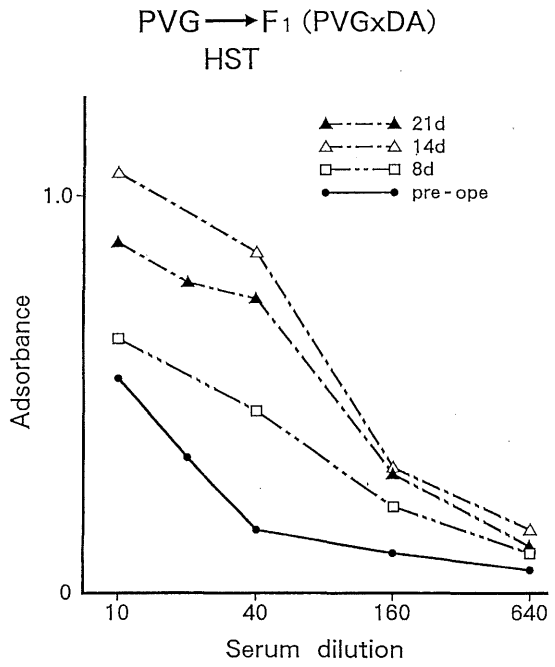

Fig. 4. Serum class I antigen in spleen- transplanted rats.
F1 hybrid rat given a PVG spleen graft. Sera were taken at various days after operation. The solid line in the figure shows the titration curve of class I antigen in the serum of F1 rats prior to operation.

PVG spleen grafting to F1 hybrid rats elevated the recipient $RT1A^a$ antigen level to several times that in normal F1 rats. The MHC haplotype of the donor spleen graft was $RT1A^c$, which does not contain any $RT1A^a$. Moreover, rejection does not occur in this combination. Therefore, the increase in serum antigen titer is due to the destruction of the host tissue membrane by spleen T cells via a GVH reaction (Fig.4).

DISCUSSION

The present study shows 1) replacement of DA liver by PVG lowered serum level of $RT1A^a$ antigen to approximately 70% of that in normal DA serum shortly after liver transplantation. However, the titer increased gradually to a level exceeding that of normal DA serum. 2) PVG spleen transplantation into F1 rats resulted in the early death of all rats due to typical GVH disease. The $RT1A^a$ antigen titer in the F1 recipient increased to several times that in normal F1 serum throughout the observation period. 3) Untreated LEW recipients of DA liver died at 9.8±1.1 days due to acute liver rejection. In these recipients, the serum $RT1A^a$ titer increased to several times that of normal DA serum. Deletion of $CD8^+$ or $CD4^+$ T cells by anti-CD8 or anti-CD4 MAb in LEW rats prior to liver grafting prolonged survival for up to 26.8±8.4 and 35.6±17.9 days, respectively. In these recipients, the serum titer of class I antigen was not elevated and there were no

differences between the two. 4) Hepatectomy in combination with Cyclosporin treatment in normal rats caused an increase in serum class I antigen.

It has been shown in many reports that class I antigen is present at very low concentrations in the serum of various animals[1,3,18,24]. However, its physiological role and origin remain unknown[11,25]. Faber and Spencer[19] have shown that the liver in the DA rat strain contains large amounts of a water-soluble RTIA class I molecule with a discrete heavy chain approximately 5 kD smaller than the menbrane-bound form. Kamada[9] has shown that DA liver-derived class I antigen appears in the circulation of recipient rats within 24 hr of liver transplantation and persists at a high titer thereafter. However, it remains unknown how much the liver contributes to the presence of class I antigen in the serum of normal animals.

The present finding that replacement of DA liver by PVG lowered the serum level of $RT1A^a$ antigen to approximately 70% of that in normal DA serum shortly after liver transplantation sugggests that the liver is the principal source of serum class I antigen and secretes it into the circulation irrespective of mechanical destruction such as that caused by rejection.

Another interesting finding is that a transient decrease in serum class I antigen titer is rapidly compensated, This is due presumably to the consequence of hyper-secretion of $RT1A^a$ by proliferating host lymphocytes during the HVG reaction. This hypothesis is supported by the *In vitro* studies of culture supernatants of cell lines and mitogen-stimulated peripheral blood lymphocytes, showing that these supernatants contain water-soluble HLA-A and B molecules with 40 kD heavy chains[15]. Interestingly, the regenerating hepatocyte is likely to secrete RT1A-class I antigen into the circulation shortly after hepatectomy. It is a well established fact that the liver has a potent regenerative capacity after its dissection and that a hepatectomized liver returns from one half of its volume to normal size within 1 month. It is also a well-known fact that the potent immunosuppressants, Cyclosporin and FK-506, augment the regeneration rate[4,13]. The result shows that liver regeneration begins shortly after liver resection and that the regenerating hepatocyte secretes more class I antigen than does normal liver hepatocytes during the early phase after liver resection.

Allogeneic rejection is mediated primarily by T cells[20]. *In vitro* studies demonstrate that the $CD8^+$cytotoxic T cells are class I MHC- reactive T cells, and that $CD4^+$ helper T cells are class II-reactive T cells[23]. The effector arm of rejection is mediated principally by $CD8^+$ T cells that target class I molecules of cell membrane-bound glycoproteins and produce liver damage. Therefore, it is

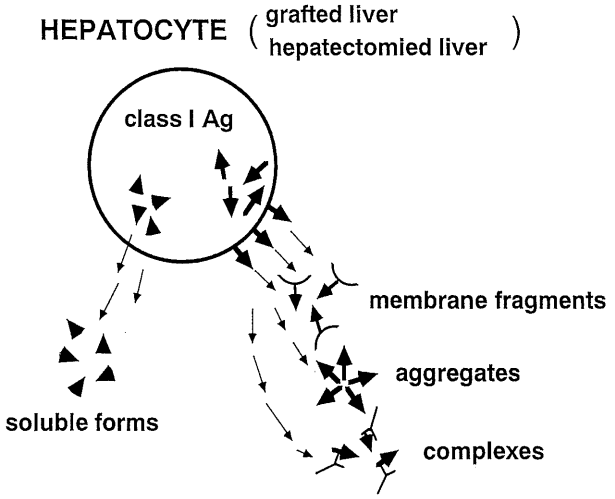

Fig. 5. Schematic representation of origin and types of serum class I antigens following liver rejection or hepatocyte regeneration.

speculated that deletion of $CD8^+$ cells causes a decrease in the serum level of class I antigens in LEW recipients of DA liver allografts. However, this was not demonstrated in this study. The reason why class I antigen is suppressed in MAb treated LEW rats may be due to the decrease in liver damage and hepatocyte regeneration.

The serum class I antigen in organ transplanted rat is anticipated to consist of several forms of class I antigen, including the soluble type, those in immune complexes, aggregates and the membrane-bound fraction (Fig.5). We[21] have shown previously by gel filtration that serum of LEW-recipient rats of a DA liver graft include two types of class I antigen. One is a soluble class I antigen with a molecular weight of 35–40 kD and the other may be a membranous or aggregated form of class I antigen with a molecular weight greater than 200 K daltons. Thus, it is proposed that soluble class I antigen is autonomously secreted from cells such as hepatocytes or lymphocytes irrespective of liver rejection. In contrast, the membranous, immune complexed or aggregated forms of class I antigen are produced as a consequence of liver rejection. Therefore, we have to distinguish the serum level of each form of class I antigen to determine more precisely the role of serum class I antigen following organ transplantation. This subject is now under investigation.

In conclusion, the liver is the principal source of serum class I antigen in rats. Liver rejection causes an increase in donor liver- derived class I antigen titer. $CD8^+$ T cells may not contribute to the elevation of serum class I antigen during liver rejection. Regenerating hepatocytes secrete class I antigen into the circulation. Serum class I antigen of host tissue origin increases during graft- versus- host reactions. This suggests that monitoring

of serum class I antigen changes after organ transplantation helps diagnose liver rejection, liver regeneration and graft-versus- host reactions. Finally, separate analysis of soluble and non-soluble form of serum class I antigen is required to determine more precisely the role of serum class I antigen after organ transplantation and liver regeneration.

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