DEMONSTRATION OF CLASS II MAJOR HISTOCOMPATIBILITY COMPLEX ANTIGENS IN KUPFFER CELLS ISOLATED FROM LONG-TERM SURVIVING RAT LIVER ALLOGRAFTS

KENICHI TERAMOTO, KENJI KANEDA, KENJIRO WAKE and NAOSHI KAMADA

1Department of Experimental Surgery, National Children's Medical Research Center, Taishido, Setagayaku, Tokyo 154, and
2Department of Anatomy, Faculty of Medicine, Tokyo Medical and Dental University, Yushima, Bunkyoku, Tokyo 113, Japan

ABSTRACT
Kupffer cells, which are normally negative to class II major histocompatibility complex antigens, express such substances in transplanted livers. To reveal the frequency of class II-positive Kupffer cells in early and late times after grafting, we isolated nonparenchymal cells from the grafts and examined the positive cells immunocytochemically. Orthotopic liver transplantation was performed in a DA-into-PVG nonrejector combination of the rat. We injected 0.81 μm-latex particles intravenously 24 h before the preparation of isolated, adherent nonparenchymal cells. The cells were centrifuged and stained with anti-donor (F17-23-2) and recipient (P7/7) class II monoclonal antibodies. We identified Kupffer cells by the phagocytosis of latex particles and counted reaction-positive Kupffer cells as well as negative ones. The proportion of donor class II-positive cells among the total Kupffer cells was 21%, <1% and <1% at 14 days, 110 days and 302 days, respectively, while that of recipient ones was 35%, 18% and 10%, respectively. Mixed Kupffer cell/lymphocyte reaction demonstrated that recipient class II antigens were expressed on Kupffer cells at both 14 and 110 days. These results indicate that Kupffer cells, not all but a part of them, gain the expression of class II antigens after transplantation and that the proportion of positive cells becomes high in an acute phase (at 14 days), then decreases and persists until 110 and 302 days. The present study also confirms the replacement of donor by recipient Kupffer cells.

In transplanted liver grafts, cellular infiltration becomes manifest at 4 days and reaches a peak at 2 weeks after surgery (4, 7). It consists of many resident-typed macrophages, i.e., Kupffer cells, which often migrate into the space of Disse (7). Several immunohistochemical studies (2, 9) have revealed that, in long-term surviving liver allografts, donor Kupffer cells are completely replaced by recipient ones between 15 and 30 days.

In a normal liver, class II major histocompatibility complex antigens, main factors in initiating a T cell immune response, are expressed only on interstitial dendritic cells, but not in the sinusoidal cells (9). In a transplanted liver, however, class II antigen-expressing cells appear inside the liver lobule, as the degree of cellular infiltration in the graft increases. They are considered to be Kupffer cells, but class II induction on sinusoidal endothelial cells cannot be excluded (9).

To demonstrate class II-positive Kupffer cells more clearly and reveal their frequency in early and late times after grafting, we have done in this study the immunocytochemical staining of isolated nonparenchymal cells, using anti-donor and recipient class II monoclonal antibodies. Before isolation, we have injected intravenously 0.81 μm-latex particles to differentiate Kupffer cells from nonphagocytic dendritic cells and endothelial cells. Sinusoidal
endothelial cells have a certain degree of phagocytic activity but do not engulf 0.81 μm-particles in vivo (1). Since infiltrating macrophages in the liver graft from 2 days to 2 months usually show the peroxidase distribution of resident-typed macrophages (7), we call here generally the obtained macrophages Kupffer cells. We have further performed mixed Kupffer cell/lymphocyte reaction to support the class II antigen expression on isolated Kupffer cells from the graft. Some of the observations described here was preliminarily reported at the International Congress of Transplantation in San Francisco (10).

MATERIALS AND METHODS

Animals

Inbred DA (RT1b) and PVG (RT1a) rats, 8–12 weeks of age, were purchased from OLAC, Bicester, Oxfordshire, U.K.

Surgical Procedure

Orthotopic liver transplantation (OLT) was performed by the cuff techniques (6), with DA rats as donors and PVG as recipients. Liver grafts in this combination are accepted permanently without requirement for immunosuppressants. Animals were sacrificed 14, 110 and 302 days after surgery.

Preparation for Kupffer Cells

One day before Kupffer cell isolation, 0.05 ml/100 g body weight of 0.81 μm-latex particles (Difco, MI, U.S.A.) were injected intravenously into PVG recipients of DA liver grafts. Liver cells were isolated according to the method of Seglen (8). The liver was perfused first by Ca(−), Mg(−)-Hanks’ balanced salt solution (HBBS) and then by 0.05% collagenase IV (Sigma, MO, U.S.A.) in HBBS via a portal vein for 30 min. After mincing, it was further incubated with the same collagenase solution at 37°C for 30 min with stirring. Thus resultant cell suspension was centrifuged three times at 50 g for 2 min and the supernatant was used for purification of nonparenchymal cells. This suspension was re-centrifuged twice at 50 g to remove residual hepatocytes. It was then centrifuged twice at 500 g for 3 min to separate nonparenchymal cells from debris. Nonparenchymal cells were plated in Falcon flasks and incubated at 37°C for 24 h under 5% CO2:95% O2. The culture medium consisted of RPMI-1640 (Gibco, NY, U.S.A.) containing 100 U/ml penicillin, 10 μg/ml streptomycin, 5 μg/ml fungizone, 50 mM 2-mercaptoethanol and 10% fetal calf serum (FCS; Gibco, NY, U.S.A.). It was changed twice during the incubation. After incubation, the culture medium was replaced by cold HBSS. Then the flasks were incubated at 4°C for 45 min. Some of adherent cells were spontaneously released from the glass surface. The remnants were removed by vigorous pipetting.

Immunocytochemistry

Adherent nonparenchymal cells purified from grafted livers were stained with monoclonal antibodies using the avidin/biotin peroxidase complex method. The cell smears were dried for 30 min, fixed in cold acetone for 30 min and washed 3 times in phosphate-buffered saline (PBS). Endogenous peroxidase was blocked by 5 mM periodate solution. The slides were overlaid with mouse monoclonal antibody F17-23-2 (anti-RT1b class II, Serotec, Bicester, U.K.) or with rat monoclonal antibody P7/7 (anti-RT1b class II, Serotec, Bicester, U.K.). After washing 3 times, the slides were incubated for 30 min with biotinylated anti-rat IgG (H+L) (Vector Lab., CA, U.S.A.) or biotinylated anti-mouse IgG (Vector Lab., CA, U.S.A.). After washing in PBS, they were incubated in avidin-conjugated horseradish peroxidase for 60 min and diaminobenzidine was added. After washing in PBS, they were counterstained with hematoxylin. We counted 100–200 cells with each specimen and calculated the proportion of reaction-positive cells among Kupffer cells.

Mixed Kupffer Cell/Lymphocyte Reaction

Responder splenic T cells, 2.5×10⁵, prepared from normal DA or PVG spleen cell suspensions by passage through nylon wool columns (Wako, Osaka, Japan), were incubated with 1×10⁵–5×10⁵ mitomycin-C treated adherent nonparenchymal cells which were collected 14 days or 110 days after transplantation. Incubation volumes were 200 μl in 96 well, flat-bottomed microculture plates (Corning, NY, U.S.A.). The culture medium consisted of RPMI-1640 containing 100 U/ml penicillin, 50 μg/ml sodium pyruvate, 1 μg/ml MEM non-essential amino acids (Gibco, NY, U.S.A.) and 5% FCS. After 72 h-incubation at 37°C in a humified atmosphere of 5% CO2:95% air, the cultures
were pulsed with 1 Ci [3H-methyl]thymidine ([3H]dR, specific activity 6.7 Ci/nmol, Amersham, U.K.) and incubated for a further 18 h. Cells were then harvested onto glass fiber filter paper strips by an automatic cell harvester (Flow Laboratories, MD, U.S.A.), and radioactivity was counted in a liquid scintillation spectrometer (Beckman, CA, U.S.A.). Splenic T cell activity, or stimulation index, was calculated using the following formula:

\[
\text{Stimulation index} = \frac{\text{cpm of culture with added adherent nonparenchymal cells}}{\text{cpm of culture of responder T cells alone}}
\]

RESULTS

Immunocytochemistry

Isolated, adherent nonparenchymal cells consisted of latex particle-phagocytosing and nonphagocytosing cells. Most of these phagocytes had a kidney-shaped nucleus, an irregular cell contour and abundant cytoplasm which contained a few to a large number of latex particles (Fig. 1, E and F). Such features are characteristic of macrophages and monocytes. Since these two cells are difficult to be differentiated from each other on the smear and the monocytes occupies only 5–10% of mononuclear phagocytes as counted histologically at day 14 (unpublished data), we call these phagocytes generally Kupffer cells hereafter. The other phagocytes were identified as polymorphonuclear leukocytes from the shape of the nucleus. Nonphagocytosing cells (Fig. 1F) are considered to be dendritic cells and contaminating endothelial cells or lymphocytes which were often attached to the Kupffer cells.

At 14 days after liver grafting, 35% of the Kupffer cells were stained with P7/7, the anti-RT1c (recipient type) class II monoclonal antibody (Fig. 1A), and 21% of them were positive with F17-23-2, the anti-RT1a (donor type) class II monoclonal antibody (Fig. 1B). There was no morphological difference between P7/7-positive and F17-23-2-positive Kupffer cells. At 110 days, the proportion of P7/7-positive cells among total Kupffer cells was 18% (Fig. 1C), while that of F17-23-2-positive cells was less than 1% (Fig. 1D). At 302 days, P7/7-positive cells occupied 10% and F17-23-2-positive cells were virtually undetectable (data not shown). We also observed a small population of class II-positive non-phagocytes (Fig. 1F) with variable sizes.

Mixed Kupffer Cell/Lymphocyte Reaction

The number of adherent nonparenchymal cells recovered from OLT rats at 14 days was much greater than that from 110 days postoperative animals. Although proliferation of DA splenic T cells was not stimulated with less than 1 × 10^4 adherent nonparenchymal cells obtained at these time periods, stimulation index rose to 2.0 at day 14 and 4.4 at day 110 with 5 × 10^4 stimulator cells and reached 25.0 at day 14 with 5 × 10^5 cells (Fig. 2, A and B). In contrast, T cells from the recipient strain PVG showed only a slight response against the stimulator cells from transplanted DA liver at either time.

DISCUSSION

This is the first report to indicate immunocytochemically the expression of class II antigens on isolated Kupffer cells from liver allografts. By preinjecting 0.81 μm-latex particles in the graft and using isolated nonparenchymal cells, we have demonstrated class II-positive Kupffer cells as well as negative cells, the latter of which are particularly difficult to be identified in histological sections. The present study indicates that Kupffer cells gain class II antigen expression as an inflammatory reaction advances in the graft, but that not all of them become positive; 21% of them were positive with donor class II antigens, 35% positive with recipient ones and the remaining 44% of the cells were negative at 14 days when cellular infiltration reached a peak. It is also noted that the expression of class II antigens on Kupffer cells persisted, although the frequency decreased, as long as 110 days and 302 days; such positiveness is considered to reflect the presence of an inflammatory reaction remaining in the graft at these times. This result is consistent with our previous observation that anti-donor class II antibodies appear in the recipient serum after liver grafting and persist at a high level for some weeks (4, 5). Such antibodies are considered to participate in promoting the general tolerance, which is induced by anti-class I antibodies, by the enhancement mechanism (4, 5). The present immunocytochemical study gives no quantitative information about the amount of class II antigens of the cell. It is reported, however, that the total amount of the antigens in the liver graft correlates well with the
Fig. 1 Immunoperoxidase staining of isolated, adherent nonparenchymal cells from liver grafts by monoclonal antibodies to rat class II RTI antigens. A, C and E are stained with monoclonal anti-recipient class II antibody P7/7, and B, D and F with monoclonal anti-donor class II antibody F17-23-2. Nuclei were counterstained with hematoxylin. A and B: day 14 post-grafting; C to F: day 110. Arrows in A to C indicate reaction-positive macrophages. Phagocytozed latex particles (asterisks) are seen inside the positive cells (double arrowheads) and negative ones (arrowheads) in E and F. The positively stained cell (arrow) in F does not contain latex particles. A-D, ×140; E and F, ×930
Fig. 2 Mixed Kupffer cell/lymphocyte reaction. $2.5 \times 10^5$ normal PVG (○-○) or DA (△-△) splenic T cells as responders were mixed with $1 \times 10^3$-$5 \times 10^5$ mitomycin-C treated adherent nonparenchymal cells separated from 14 days or 110 days postoperative OLT rats. A: Stimulators were obtained from 14 days postoperative OLT rats (2 W OLT-KC). B: Stimulators were from 110 days postoperative OLT rats (16 W OLT-KC). Values are the means of three experiments. Bars, standard deviations.

frequency of class II-positive cells (9).

We have further revealed here that donor Kupffer cells could be replaced by recipient Kupffer cells in the long-term surviving liver grafts. At 110 days, the Kupffer cells positive against donor-type antigens disappeared, while a significant number of Kupffer cells positive against recipient cells persisted. Although not all Kupffer cells expressed class II antigens as shown here and therefore the origin of such negative cells could not be attributed to donor or recipient specific anti-class II reagents, the present result favors the replacement of donor by recipient Kupffer cells. Settaf et al. (9), using the same combination (DA-into-PVG) and staining liver graft sections in situ with monoclonal anti-class II antibodies, have demonstrated that the structures positive to donor class II antigens appear from day 3, increase in number up to day 15 and, by day 30, have disappeared from the hepatic lobules, while a large number of class II-positive recipient cells can be detected at day 30. Our present results are in accordance with their observations. Gassel et al. (2) also showed similar results in other combinations such as BN-into-LEW and DA-into-LEW.

The data of mixed Kupffer cell/lymphocyte reaction also supports the replacement of Kupffer cells. More than $5 \times 10^5$ adherent nonparenchymal cells from both 14 and 110 days postoperative OLT rats induced a significant proliferation of DA T lymphocytes. In this study, the stimulation index at day 14 was lower than that at day 110 with $5 \times 10^4$ stimulator cells (Fig. 2) contrary to the immunocytochemical findings of higher frequency of class II-positive Kupffer cells at day 14. One possible reason for this is that much more peripheral leukocytes such as lymphocytes are included in the adherent nonparenchymal cell fraction obtained at day 14 than at day 110, which lowered the overall proportion of class II-positive cells. It is unknown, however, why PVG lymphocytes were not stimulated by Kupffer cells from the graft at 14 days in spite of the presence of DA class II-positive Kupffer cells.
Several studies have suggested that Kupffer cell replacement will remove an important source of class I antigens from the liver and promote its long-term survival (2, 3, 9). Absence of class II alloantigenic stimulation from Kupffer cells in the graft as a result of replacement may also assist the acceptance of the graft.

Received 15 April 1991; and accepted 2 August 1991

REFERENCES