Cyclosporine resistant effector cells in rabbit skin allografts*

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Summary. Histoincompatible skin grafts in rabbits treated with cyclosporine can permanently engraft but show a transient mononuclear cellular infiltrate. This transient cyclosporine-resistant infiltrate consists of cells which are sensitive to steroids, radiation and cryopreservation. They have the same ACM-1 phenotype and the same characteristics as cyclosporine-sensitive cells.

Key words. Cyclosporine; skin transplantation; bone marrow transplantation; rabbit; graft-versus-host-disease.

Cyclosporine is the most specific immunosuppressive agent used today following organ transplantation[1]. Despite its potential, graft rejection or graft-versus-host-disease (GvHD) can occur in the presence of 'adequate' blood levels. These observations suggest that both cyclosporine-sensitive and cyclosporine-resistant effector cells in organ graft rejection and in GvHD must exist. The question remains whether resistant and sensitive cells are of different cell types or whether they recognize different targets. In this study we tried to characterize the cyclosporine-resistant effector cells in rabbit skin grafts.

Materials and methods. Animals. Outbred adult New Zealand White male and female rabbits were used as recipients and Red Burgundy rabbits as skin graft donors. These two strains are strongly histoincompatible[2]. Animals were housed in single cages and kept on pellet diet and water ad libitum. They were inspected daily for their clinical status.

Skin and bone marrow grafts. The techniques have been described in detail[3]. Full thickness 1 × 1-cm skin grafts from one donor were transplanted on the shaved back of the recipients, dressed with an antibiotic gauze and covered with adhesive tape. All grafts were inspected three times a week and their degree of induration was recorded.

For autologous bone marrow transplantation, bone marrow was harvested from the iliac crests and from the femurs under general anesthesia with Hypnorm®. Approximately 1×10^8 nucleated cells/kg were collected and layered on Ficoll-Ronpacon (d = 1.09) to separate mononuclear cells. Cells were given either fresh the same day or after cryopreservation a few days later (see study design). Cryopreservation was performed in TC 199 containing 5% autologous plasma and 10% DMSO in the final concentration. Cells were frozen using a biological automated freezer (Planer 2000 R) at a rate of −1 °C/min. Total body irradiation (TBI) was performed using a single 60′ Co source. 12 Gy TBI midline tissue doses were applied at a rate 45 cGy/min.

Cyclosporine. Cyclosporine was kindly provided by Sandoz Ltd, Basel. Ampoules containing 1 mg/ml, dissolved in miglyol, were used. Cyclosporine treatment was always started the day before the skin graft (day −1) and continued for 60 days at the dose outlined in the table.

Study design. Nine groups of animals with 5–10 in each group were studied (table). Groups A–E were given a skin graft only, with different schedules of cyclosporine treatment. In groups F, G, H skin grafting was performed one day after 12 Gy TBI followed by autologous bone marrow rescue. The bone marrow was given fresh (G; in this group the bone marrow was aspirated and reinjected after TBI the same day) or cryopreserved (F, H). In addition, animals did (G, H) or did not (F) receive cyclosporine at the same dose as animals in group B.

Histological examination. Rabbits are fragile laboratory animals. Repeated general anesthesia after TBI and bone marrow transplantation for biopsies is not tolerated, especially since cyclosporine adds significant toxicity. A group of 8 additional animals was therefore specifically studied for serial skin biopsies. All animals were transplanted with six 1 × 1-cm grafts from one donor, distributed over their backs. All donors and recipients in this experiment were typed for immunoglobulin allotypes. In each group two recipients were found to be immunoglobulin allotype-matched and two were immunoglobulin allotype-mismatched. Four animals were given no cyclosporine while 4 animals were given 15 mg/kg s.c. daily like the animals in group B. These transplanted skins were explanted in toto on days 5, 10, 15 in animals without cyclosporine (at day 20 the skin transplants had already been rejected) and on days 5, 15, 25, 40 in cyclosporine-treated animals. Explanted grafts were then cut in half and either processed for routine histological staining or frozen for immunohistological examination. Frozen sections were incubated with an anti-T-cell monoclonal antibody for the determination of T-cells (ACM-1) (kindly provided by Dr F. Adler, Memphis, Tennessee). This antibody recognizes T-cells but not B-cells; however, it cross-reacts with some granulocytes and monocytes[4].

Results. Infiltration of transplanted skin grafts occurred in all animals as described before[3,5]. It varied markedly between the individual animals and between the different groups (figs 1 and 2). Complete rejection of all grafts occurred in the control groups A and F. TBI followed by autologous bone marrow transplantation did not prevent rejection. As previously published[3], 15 mg/kg are needed to achieve sustained engraftment (group B). At a lower dose, 10 mg/kg (group C) two animals rejected their skin graft. Duration and intensity of infiltration was marked in this group with the lower dose of cyclosporine. However, an increase of the dose of cyclosporine did not prevent the transient skin infiltration (group D); toxic reactions were more pronounced (6).

Skin infiltration was mild in groups E, G, H. The addition of a high dose of steroids almost completely abolished the infiltration. Toxicity, however, was again severe leading to the death of all animals within 30 days. A mild infiltrate was observed in the animals given TBI and it was almost completely absent in the group rescued with cryopreserved bone marrow (H).

On histological examination the degree of infiltration paralleled the clinical signs, it consisted mainly of mononuclear cells. On immunohistological examinations the mononuclear

Study design

Group	No.	Cyclosporine therapies Dose (mg/kg)	Duration	Additional therapy
A	5	–	–	Control group
B	10	15	60 d	
C	10	10	60 d	
D	6	30*	60 d	
E	5	10	60 d	+ Prednisone 20 mg/kg i.v. daily
F	10	–	–	TBI 1200 rad + cryopreserved autologous BMT, control group
G	10	15	60 d	TBI 1200 rad + fresh autologous BMT
H	10	15	60 d	TBI 1200 rad + cryopreserved autologous BMT
I	8	see text	60 d	Serial biopsies

*reduced to 15 mg/kg after 6 days

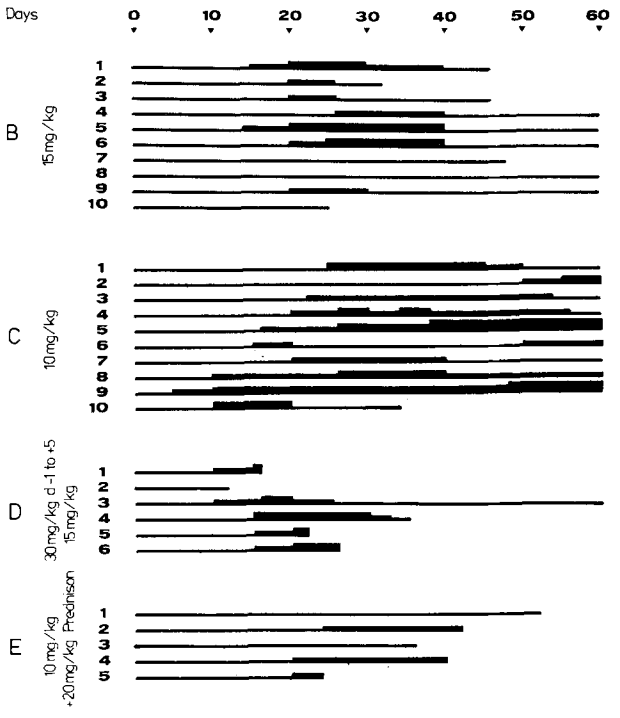

Figure 1. Degree of skin infiltration and survival in skin grafted cyclosporine treated rabbits (groups B, C, D, E). No infiltration or minimal infiltration ——, infiltration 50% ▬, infiltration less than 50–75% ▬, skin rejected ■.

cells stained with the monoclonal antibody ACM-1. In animals not treated with cyclosporine, the infiltrating cells were also positive with ACM-1. In untreated animals, the infiltrate continued to increase until rejection. In cyclosporine-treated animals, histological examination paralleled the clinical observations. An infiltration of mononuclear cells, positive with ACM-1, is seen between days 15 and 40.

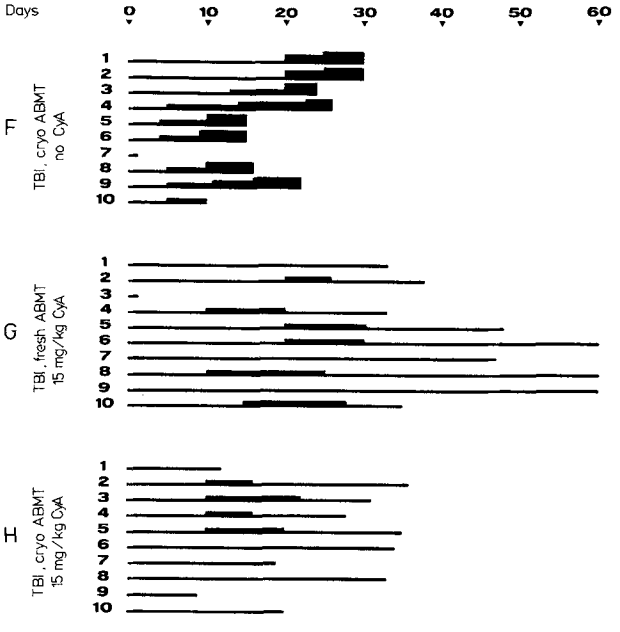

Figure 2. Degree of skin infiltration and survival in skin grafted cyclofollowing TBI and autologous BMT (groups F, G, H). No infiltration or minimal infiltration ——, infiltration 50% ▬, infiltration less than 50–75% ▬, skin rejected ■.

These cells are no longer present beyond day 40. The same pattern of infiltrate, clinically, histologically and immunohistologically is seen in animals matched or mismatched for immunoglobulin allotypes.

Discussion. Cyclosporine clearly allows sustained engraftment of histoincompatible skin grafts in rabbits. However, as reported earlier, these skin grafts show a transient infiltrate of mononuclear cells. Similar infiltrates are observed in kidney, heart and lung allografts[7]. In addition, rejection or GvHD can occur despite apparently 'adequate' levels. These findings suggest the presence of cyclosporine-resistant effector cells in allograft reactions[5]. They support a concept that graft rejection is a network of multiple effector cell mechanisms[8]. In this study we tried to characterize these cyclosporine-resistant effector cells. There are some limitations. We do not know whether these cells are indeed cyclosporine-resistant or only less sensitive. Toxicity[6] prevents a dose escalation study. However, the results in group D at least suggest that more cyclosporine does not abolish such infiltrates. The second limitation is the poor characterization of rabbit lymphocytes. In contrast to those of mice, rats and man lymphocyte subsets are still ill-defined. The antibody used, ACM-1, is a broad reacting pan-T monoclonal antibody. We cannot exclude an infiltration by, for instance, NK-cells or another cell type. Histomorphology, however, shows that the cells are not granulocytes, and they do not exhibit characteristics of large granular lymphocytes.

The degree of infiltration was identical in animals matched or mismatched for immunoglobulin allotypes. This absence of a difference suggests that humoral immune mechanisms are not likely to be responsible for the cyclosporine-resistant infiltrate.

We could clearly define the influence of certain maneuvers on this cellular infiltrate. A high dose of prednisone, synergistic to cyclosporine in clinical transplantation, reduced this infiltrate and documents that these cells are sensitive to steroids. TBI reduces the transient infiltrate. The most marked reduction was seen in those animals given TBI and cryopreserved bone marrow followed by cyclosporine (H). The skins stayed smooth and flat in most animals throughout the course of the experiment and almost no transient skin infiltrate was seen. These findings suggests that radiation reduces the number of infiltrating cells. A few cells most likely derive from the infused bone marrow, since cryopreservation further reduces the infiltrate. The influence of cryopreservation fits the previous observations[3], that GvHD is delayed in rabbits after infusion of a cryopreserved allogeneic bone marrow. We know today that rabbit T-lymphocytes form spontaneous rosettes[9] at lower temperatures and that the manipulations performed for bone marrow cryopreservation selectively induce a T-cell-depletion of the bone marrow.

In summary, we have shown in this study that cyclosporine-resistant cells transiently infiltrating skin grafts are bone marrow derived, radiosensitive, cryosensitive, steroidsensitive and of ACM-1 phenotype. We conclude that cyclosporine-resistant and cyclosporine-sensitive effector cells are of the same cell type and cannot be distinguished by available methods. We speculate that they are different in their recognition of antigenic epitopes on skin target cells.

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Multiple organ-reactivity of monoclonal autoantibodies to mouse erythrocytes

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Summary. Autoantibodies reacting with bromelain-treated autologous mouse red blood cells (Br-MRBC) are spontaneously produced by normal mice. In order to understand the biological significance of these autoantibodies, anti-Br-MRBC monoclonal autoantibodies have been prepared and studied for reactivity with a panel of frozen tissue sections from organs of normal mice by direct immunofluorescence. It has been found that the anti-Br-MRBC monoclonal autoantibodies are polyspecific, since they react with cells in multiple organs.
Key words. Br-MRBC; monoclonal antibodies; autoimmunity.

Lymphoid tissues of normal mice contain cells that secrete autoantibodies to autologous mouse red blood cells (MRBC) treated with bromelain (Br), a proteolytic enzyme[1,2]. The biological significance of anti-Br-MRBC autoantibodies is not known, but this phenomenon seems to constitute a model of spontaneous autoimmunity for at least two reasons: first, the bromelain treatment does not create a new antigen, but only reveals a 'hidden' autoantigen[3]; second, the development of anti-Br-MRBC antibodies is inhibited by suppressor T lymphocytes and by the presence of the autoantigen[3-6], as in the case of other models of autoimmunity.

Either a cross-reacting environmental antigen or a self-antigen present in the body are thought to activate the immune response to autologous Br-MRBC. Several arguments, however, favor the latter possibility; for example, germ-free and pathogen-free mice have the same number of cells making antibodies to Br-MRBC as conventionally reared mice of the same strain[3]; moreover, the antigen binding to Br-MRBC antibodies has been isolated both from erythrocytes and serum[7]; finally, anti Br-MRBC antibodies also bind to the Fc portion of the IgG molecule[8].

To know whether normal mice express other autoantigens capable of binding to Br-MRBC antibodies, we have prepared anti-Br-MRBC monoclonal autoantibodies, using peritoneal cells as fusion partners[5], and we have studied their reactivity with a panel of tissue sections from different organs of normal mice.

Materials and methods. Peritoneal cells (PC) from 2–3-month-old inbred Balb/c female mice were collected by injecting into the peritoneal cavity 5 ml of RPMI-1640 (Flow Laboratories, Rickmansworth, U.K.) supplemented with 1% fetal bovine serum (FBS), and 5 U/ml heparin. The cells were pooled, washed three times at 400 g for 8 min at 4 °C, and then 15×10^6 PC were fused with 7.5×10^6 P3.X63.AG8.653 nonsecretor myeloma cells by using 1 ml of 50% polyethylene glycol (MW 1000) at neutral pH. Fused cells were washed and suspended in growth medium (RPMI-1640 with 10% FBS, 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.2 µg/ml fungizone) containing hypoxanthine (100 µM), aminopterin (0.4 µM), and thymidine (16 µM) (HAT medium), and seeded in 0.2 ml volume at 2×10^5 cells/well in 96-well plates. Hybrids were fed by replacing 100 µl of HAT medium twice a week for the first two weeks, and then cultured for five more days in HT medium.

Outgrowing hybrid cells were tested for the production of autoantibodies to Br-MRBC by a plate modification of the Jerne and Nordin plaque-forming cell (PFC) assay[9], using, as target, mouse red blood cells (MRBC) obtained by bleeding Balb/c mice from the axillary vein under ether anesthesia, and immediately treated with bromelain (Serva Feinbiochemica, Heidelberg, FRG), as previously reported[6]. The cultures producing autoantibodies to Br-MRBC were cloned twice by limiting dilution on feeder cells consisting of 5×10^3 peritoneal cells per well collected from normal Balb/c mice. The hybrid cell clones producing autoantibodies to Br-MRBC were expanded and the culture supernatants were precipitated with ammonium sulphate (50% of saturation) and passed through anion-exchange cromatography column. The fractions showing hemolytic activity in the presence of complement were pooled and labeled with fluorescein isothiocyanate (FITC; Sigma Co., St. Louis, Mo., USA) by dialyzing the antibody solution against a 10-volume solution of FITC (100 µg/ml) in 0.05 M bicarbonate buffered saline, pH 9.2, at 4 °C overnight. The reaction was stopped by changing the dialysis buffer to 0.02 M phosphate-buffered saline pH 7.0 (PBS). The free FITC was removed from the conjugated by chromatographing labeled samples on Sephadex G-25 in PBS. The fluorescein-conjugated monoclonal antibodies were then tested for reactivity with a panel of frozen tissue sections of normal organs from Balb/c mice by incubating each section with a 1:20 dilution of fluorescent antibody in PBS for 1 h at room temperature and then by washing three times in PBS. Tissue sections were from thyroid, salivary glands, adrenals, ovary, testis, epididymis, heart, lung, pancreas, kidney, liver, stomach, intestine, pituitary, thymus, and lymph node; the monoclonals were also tested on smears of acetone-fixed bone marrow cells. A fluorescein-labeled IgM monoclonal antibody to Coxsackie B3 virus (Garzelli et al., manuscript in preparation) was used as negative control.

Results. Peritoneal cells from normal Balb/c mice were fused by polyethylene glycol with mouse myeloma cells and the fusion products were distributed into a microtiter plate. Of the 96 wells that were seeded, 44 showed cell growth. These cultures were tested for autoantibodies to Br-MRBC by hemolytic PFC assay. Two hybrid cell cultures were found to lyse bromelain-treated mouse erythrocytes in the presence of complement; they were, however, unable to lyse normal erythrocytes. These two autoantibody-producing hybrids were cloned twice by limiting dilution and two stable sub-