

CLASS I TRANSPLANTATION ANTIGENS IN SOLUTION IN BODY FLUIDS AND IN THE URINE

Individuality Signals to the Environment

BY PRIM B. SINGH,* RICHARD E. BROWN,† AND BRUCE ROSER*

*From the *Immunology Department, AFRC Institute of Animal Physiology and Genetics Research,
Cambridge Research Station, Babraham, Cambridge CB2 4AT, United Kingdom; and*

†Department of Psychology, Dalhousie University, Halifax, Nova Scotia, Canada B3H4J1

The classical class I antigens (HLA-A, -B, -C in man, H-2K, D in mouse) of the MHC are glycoproteins integrally inserted in the limiting membrane of nearly all cells in vertebrate species. The genes that encode these glycoproteins occupy the most polymorphic loci known in vertebrates. Since their glycoprotein products act as strong transplantation antigens, their polymorphism constitutes an intractable barrier to cross-matching of tissues from unrelated donors for organ grafting. The full extent of polymorphism is not completely defined, but best estimates put the likely number of alleles at up to 100 at each of three genetic loci in man, the B locus being more polymorphic than the A locus, which is much more polymorphic than the C locus (1). Similar allele frequencies are estimated for mice (2). In such outbreeding mammals this level of polymorphism ensures that in each species there are $>10^9$ possible unique class I antigenic phenotypes. The function of forced polymorphism on this scale is not understood.

The location of classical class I molecules in cell membranes and the fact that they are used by the T lymphocytes of the immune system as associative recognition molecules (3, 4) has directed attention toward immunological explanations for polymorphism. The current explanation is that MHC polymorphism in a population ensures that lethal pathogens such as viruses cannot extinguish a species by epidemic infection (5). In the absence of MHC polymorphism, mutation of the coat proteins of a virus to mimic host self-structures or to a structure that does not associate with the MHC molecules would ensure its freedom from immune attack. Thus infectious agents would purge the population of common allelomorphs and, by frequency-dependent selection, favor the rarest types. While this explanation is undoubtedly valid and explains the occurrence of MHC polymorphism in a species it does not predict the level of polymorphism required and certainly does not account for a system that can render nearly every individual in a population antigenically unique.

We now show that classical, polymorphic class I molecules in normal rats are not only secreted by hemopoietic cells into the body fluids but are constitutively excreted

This work was supported by grants from the Medical Research Council of Great Britain, the National Science and Engineering Research Council of Canada, and by a Royal Society Bilateral exchange grant. P. B. Singh was supported by an AFRC studentship and a Magdalene College Bye-Fellowship. B. Roser's present address is Quadrant Research Foundation, P.O. Box 236, Cambridge CB1 6EH, U.K.

in the urine, and that untrained rats can distinguish the smell of urine samples taken from normal donors that differ only at the class I MHC locus and therefore excrete different allelomorphs of class I molecules in their urine. Thus, the family of class I glycoproteins that are markers of the uniqueness of individuals are not, as was previously thought, sequestered within the body but are excreted into the environment where they could act as the recognition signals that regulate social interactions between individuals within a species.

Materials and Methods

Animals. Rats of the DA(*RTI^{av1}*), PVG(*RTI^c*), PVG.R1(*RTI^{r1}*), PVG-*RTI^u*, Wistar Albino, F344(*RTI^{h1}*), and (PVG × DA)_{F1} hybrid strains were bred in conventional animal houses or in a specific pathogen-free unit. AS2(*RTI^f*), and BN(*RTIⁿ*) strains were obtained from OLAC 1976 Ltd., (Bicester, United Kingdom). Some PVG-*RTI^u* animals were obtained from the Sir William Dunn School of Pathology, University of Oxford, Oxford, U.K.

For behavioral experiments, 70 PVG-*RTI^u* rats were housed in pairs in opaque plastic cages (North Kent Plastics Ltd., Dartford, U.K.) on a reversed 12:12 L/D cycle with lights off at 6.00 hours at a temperature of 20 ± 2°C. Food (CRM NUTS, Labsure irradiated diets) and water were freely available. At the beginning of the experiment, the subjects had a mean weight of 255 ± 20 g.

Enzyme-linked Immunoabsorbent Assays (ELISA). ELISAs were carried out in rigid, nonsterile, flat-bottomed, 96-well polystyrene plates (Nunc Immunoplates II, Gibco Ltd., Middlesex, U.K.). 200 µl of a 20-µg/ml solution of purified anti-class I mAb in PBS/0.1% azide, pH 7.4, was used to coat individual wells. The plates were covered and kept at 4°C until use. On the day of use excess antibody was removed and wells were post-coated up to the brim with PBS/azide containing 10% vol/vol FCS for 1 h at 4°C to block nonspecific protein adsorption sites. After 1 h the plates were washed twice with PBS + 0.5% wt/vol Tween 20 (Sigma, Poole, England) (PBST). 200 µl of a solution containing the class I MHC antigen (or a titration thereof) was then added to the appropriate wells and incubated overnight at 4°C. After three washes with PBST and three washes with PBST + 2% vol/vol FCS, 200 µl of a second noncompetitive anti-class I mAb coupled to biotin was added at 8 µg/ml and incubated for 1 h. The plate was again washed three times with PBST and three times with PBST + 2% vol/vol FCS before addition of 200 µl of a 4-µg/ml concentration of Stepavidin-coupled horseradish peroxidase (Miles Laboratories Ltd., Slough, U.K.). The plates were then incubated for 20 min at 4°C. After washing twice with PBST + 2% vol/vol FCS and twice under tap water for ~15 s each time, 200 µl of the substrate 3,3',5,5'-tetramethyl benzidine at 100 µg/ml was added. The reaction was stopped after 5 min with 50 µl of 2 M H₂SO₄ and the plates were read in a Titertek Multiscan ELISA plate reader (Flow Laboratories Ltd., Irvine, Scotland).

In the assay for detection of the A^{av1} class I MHC antigens of the DA strain the capture antibody on the plate was JY3/109 (haplotype specificity, *av1+*, *c-*, *f-*, *h-*, *l-*, *n-*, *o+*, *u-*, *b+*), while the second biotin-labeled antibody was JY1/116 (6) (haplotype specificity, *av1+*, *c-*, *f-*, *h-*, *l-*, *n-*, *o-*, *u-*, *b+*). For the detection of the A^c antigens of the PVG strain, the capture antibody was YR5/310 (haplotype specificity, *av1-*, *c+*, *f-*, *h+*, *l-*, *n-*, *o-*, *u-*), and YR5/12 (7) (haplotype specificity, *av1-*, *c+*, *f-*, *k-*, *l-*, *n+*, *o-*, *u-*) was the biotin-labeled second stage. All are rat IgG alloantibodies.

Affinity Chromatography. Class I molecules were extracted from liver cell membranes or lymph by affinity chromatography with specific anti-class I mAbs covalently coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia, Uppsala, Sweden). To prepare the membrane-bound form, DA strain rat livers were homogenized in a buffer that consisted of 0.05 M Tris-HCl, pH 7.2, 0.25 M Sucrose, 25 mM KCl, 5 mM MgCl₂, and 1 mM PMSF (Sigma) to inhibit proteolysis. Crude membranes were obtained by centrifugation at 10³ g for 20 min to remove nuclei and large debris and at 1.05 × 10⁵ g for 1 h over a 40% wt/wt sucrose cushion to sediment the membrane fraction. This was then made up to 10 mg/ml with 0.02 M Tris/HCl, pH 7.8, containing 0.5% wt/vol *n*-octylglucopyranoside (Sigma).

The solubilization was carried out for 1 h at 4°C, stirring gently throughout. The material was then centrifuged at $1.05 \times 10^5 g$ to remove debris. The RT1.A^{av1}-containing supernatant underwent affinity chromatography according to Parham (8), on a JY1/116 anti-RT1.A^{av1} affinity column which was eluted with 0.015M carbonate buffer, pH 11.0, containing 0.5% wt/vol *n*-octylglucopyranoside. The eluted fractions were concentrated using a CX30 concentrator (Millipore Corp. Bedford, MA) before running on SDS-PAGE.

To purify the soluble RT1.A^{av1} molecules, lymph was spun at $1.05 \times 10^5 g$ for 1 h so that all the lipid and lipoproteins floated and could be removed. The opalescent lymph was loaded onto the JY1/116 affinity column and washed as above, except there was no detergent in the buffers. RT1.A^{av1} was eluted with 0.5% wt/vol *n*-octylglucopyranoside in 0.015 M carbonate buffer, pH 11.0, concentrated and run on SDS-PAGE as for the solubilized membrane material.

To purify soluble RT1.A^{av1} molecules from urine, 500 ml of urine collected from eight DA animals was spun at 105,000 *g* to remove any material that may block the columns and then was treated as in the case of lymph.

(SDS-PAGE). SDS-PAGE analysis of proteins purified by affinity chromatography was undertaken according to Laemmli (9) and bands stained with Coomassie blue.

Inhibition Binding Assay. Since noncompetitive pairs of anti-class I mAb are not available for all strains of rats, the presence of soluble class I MHC antigens in five strains (AS2, BN, PVG, F344, and DA) was shown by an inhibition binding assay. Use was made of the mouse anti-rat RT1.A mAb F16.4.4 (10), which recognizes a nonpolymorphic determinant on rat class I MHC antigens. The assay consisted of inhibiting the binding of 25 μ l of a predetermined dilution of F16.4.4 to 25 μ l 1% vol/vol suspension of DA RBC by 25 μ l of the serum from the various strains. The cells were washed, harvested, and the radioactive antibody bound to them was counted using a Packard model 5210 gamma counter (Packard Instrument Co., Downers Grove, IL). The inhibition of binding of F16.4.4 to the target RBC by the various rat sera was corrected by subtraction of the mouse serum control.

Radiation Chimeras. 3-5-mo-old SPF rats received 10 Gy (1,000 rad) whole body radiation from a ¹³⁷Cs source (Gammacell 40; Atomic Energy of Canada Ltd., Kanata, Ontario) at ~ 1 Gy/min. On the same day they were given 10^8 donor bone marrow cells intravenously. In the parent into F₁ chimeras, graft-vs.-host disease was avoided by using bone marrow cells from DA rats neonatally tolerant to (PVG \times DA)F₁. Serum from all chimeras was assayed for the unshared soluble class I molecules by ELISA. Staining of peripheral blood leukocytes for class I molecules by fluorescence using mAbs and FACS 420 analysis showed the hemopoietic system of these chimeras to be >98% donor type at 9 mo after reconstitution.

Scatchard Analysis. The anti-A^{av1} antibody JY1/116 (6) and the anti-A^c antibody YR5/12 (7) were iodinated using the lactoperoxidase method (11) at a ratio of 1 μ Ci to 1 μ g of purified IgG. These antibodies were then used to measure the number of antigenic sites on the relevant rat erythrocytes. Titrated amounts of antibody were incubated with 25 μ l of a 10% vol/vol RBC suspension for 1 hr at 4°C and the cells were separated from unbound antibody by centrifugation on a 200 μ l mixture of Di-*n*-butylphthalate (eight parts) and Di-nonyl phthalate (two parts) (BDH Chemicals Ltd., Poole, U.K.). The tubes were frozen with solid CO₂ and then cut at the midpoint of the frozen oil column. The RBC pellet and the floating supernatant were counted separately in a gamma counter (model 5210; Packard Instrument Co.) to give a precise measure of bound and unbound antibody. The antibody affinity and number of antigenic sites were then calculated by the method of Scatchard (12).

Saturation Analysis. One-tenth of the amount used in Scatchard analysis, i.e., 25 μ l of a 1% vol/vol RBC suspension was then used in an antibody-binding inhibition assay using 25 μ l of neat serum of the relevant type in 12 replicates. The three reactants, ¹²⁵I-labeled antibody, serum-containing antigen, and RBC were incubated for 1 h on a shaker at 4°C to reach equilibrium. The cells were washed three times with PBS/azide + 2% vol/vol FCS, harvested onto glass fiber paper, washed again using a Skatron harvester (Flow Laboratories), and counted in a Packard gamma counter. Results were processed according to the formulas described by Ekins (13). Serum in which the amount of class I molecule was known from saturation analysis was aliquoted and stored at -20°C. This was always run in ELISA as a positive calibration control. The amount of antigen in unknown samples run in these assays could then be estimated accurately by measurement of the horizontal displacement of regression lines drawn through the linear portions of the ELISA titration curves.

Tissue Culture Methods. Cells were handled in Dulbecco's PBS with 2% FCS. PBS/2% FCS containing 0.1% sodium azide, was used for cellular RIAs and antibody-binding inhibition assays.

In experiments involving tissue culture, 10^6 cells were cultured in 1 ml of tissue culture medium in 24-well tissue culture plates (Nunc; Gibco Ltd.), in RPMI-1640 containing 25 mM Hepes, 5×10^{-5} M 2-ME, 2 mM glutamine, 0.06 mg/ml benzylpenicillin, 0.1 mg/ml streptomycin sulphate, and 5% vol/vol FCS (batch no. 001703; Sera-Lab, Crawley Down, England).

Half-Life Measurement. Four DA rats rendered neonatally tolerant of PVG antigens were bled of 3 ml of blood from the tail vein. They were then immediately injected intravenously with 5 ml of PVG serum. After 10 min the first blood sample was taken. A blood sample was then taken from one pair of rats 2 h later and from the other pair after another 2 h. The pairs were then bled alternately at 4-h intervals.

Neonatally Tolerant Rats. Rats were tolerized according to a previously published protocol (14). Briefly, DA or PVG neonates were injected intravascularly with 75 μ l of a 50% vol/vol suspension of (PVG \times DA) F_1 hybrid bone marrow cells within 24 h of birth. As adults, these rats failed to reject skin grafts from donors homozygous for the unshared antigens of the F1 for >50 d. Their lymph node cells were shown to be unreactive against donor antigens in popliteal lymph node graft-vs.-host assays (15) before they were used in experiments.

HPLC-Gel Filtration. Under nondissociating conditions, 200 μ l of PVG serum and 200 μ l of purified RT1.A^c molecules from DA urine were fractionated on a TSK G3000SW column (LKB Producter, Bromma, Sweden) running at 0.5 ml/min in PBS/azide.

Bilateral Nephrectomy. Both kidneys were exposed through a midline incision and the vascular pedicle and ureters were exposed by blunt dissection. The pedicle and ureters were ligated *en bloc* with a single silk ligature and the kidneys were removed distal to the tie. The abdominal wound was closed with two layers of continuous silk suture.

Habituation-Dishabituation Tests. PVG-RT1^a subjects were given a 15-min habituation to the test arena (an opaque plastic cage measuring 29.6 \times 23.6 \times 14.6 cm with a one-half-inch square wire mesh top that rose another 5.6 cm above the cage rim) and were then tested once per week in a habituation-dishabituation test (16) in which nine sequential 2-min odor presentations were given. For these presentations, 0.1 ml of liquid was placed on a 7-cm diameter disk of Whatman No. 1 filter paper, which was taped to the mesh top of the cage, with the center of the disk 13.5 cm from the floor of the arena. The odorized disk was replaced every 2 min, by removing the top of the cage and replacing it with another top carrying a new odorized disk. Tops and arenas were washed in 70% ethanol after each test and filter papers were used only once.

On the first three 2-min tests, water was placed on the filter paper so the subject had a 6-min habituation to the test procedure before the first urine sample was presented for the next three 2-min trials. On trial seven, the second urine sample was presented for three trials. Donor urine was collected from 10 individual PVG and 10 individual PVG.R1 males the same age as the subjects in Urimax, metabolic cages for 6 h during the day and was frozen in aliquots until used. Each subject was given two urine samples from known individuals.

The time spent rearing on the hind legs and sniffing, with the nose within 1 cm of the disk throughout each 2-min period, was recorded by an observer using a stopwatch. Each of the rats was tested in three habituation-dishabituation tests. The observer was blind to the order of testing odorant samples that were prepared by an assistant according to a random list.

Statistical Methods. Analysis of the behavioural data was done using separate randomised block (repeated measures) analyses of variance for each experiment. Post-hoc analyses were done using Newman-Keuls tests (17).

Results

Classical, Polymorphic Class I Antigens in Solution in the Body Fluids. Soluble class I antigens were first detected in the blood of normal rats by their ability to inhibit

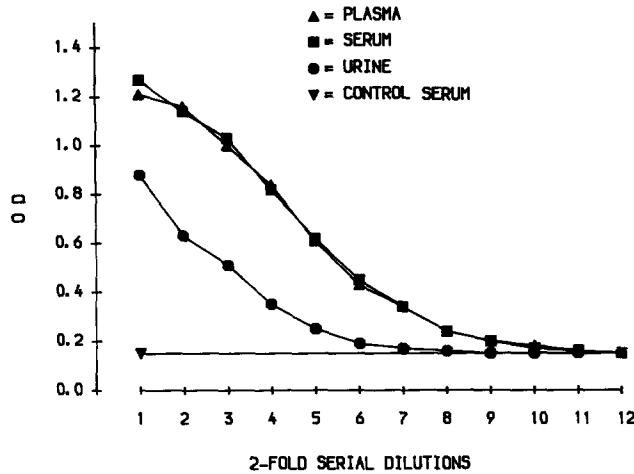


FIGURE 1. PVG urine, serum, and plasma, prepared by collecting aortic blood into chilled heparinized saline and immediate centrifugation at 4°C, was assayed for the presence of soluble RT1A^c class I antigens using the ELISA. Urine (●) contains from a half to an eighth the concentration of class I molecules found in serum (■) or plasma (▲). Control DA serum (▼) gives a small background in this assay.

target cell lysis by a cytotoxic antiserum (18). To show that they were not trace contaminants in the serum released either from damaged liver tissue or from formed elements of the blood during clotting, soluble class I molecules were detected in fresh serum, plasma, urine (Fig. 1), and lymph (19) by ELISA. Analysis of the MHC molecules, recovered from lymph by affinity chromatography, in SDS-PAGE revealed a heterodimeric molecule with a heavy chain M_r of 39×10^3 and a light chain M_r of 13×10^3 typical of β_2 -microglobulin (Fig. 2). Body fluids of the rat therefore contain authentic classical polymorphic class I antigens in solution, and fractionation of serum by gel-filtration HPLC under nondissociating conditions (Fig. 3 a) shows these molecules to be true monomers and not molecular aggregates.

Sera of five rat strains of different MHC types were examined for the presence

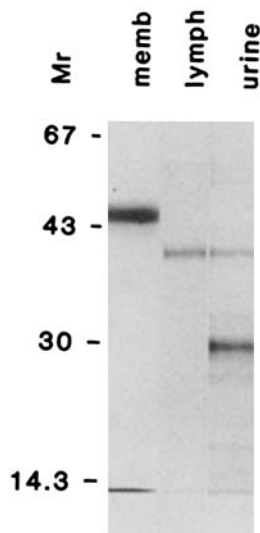


FIGURE 2. SDS-PAGE analysis of membrane-bound class I molecules and soluble class I molecules from lymph and urine. Membrane-bound A^{av1} molecules from DA liver have a heavy chain M_r of 47×10^3 and a β_2 -microglobulin light chain of 13×10^3 . The lymph molecule has a smaller heavy chain (M_r 39×10^3). The M_r of the class I heavy chain in urine is 39×10^3 as it is in the serum. The 13×10^3 M_r light chain of β_2 -microglobulin is also seen indicating that the molecules detected by ELISA are typical class I molecules. In addition, there is a major band at 30×10^3 M_r found in the preparation derived from urine that is not seen in either the lymph or membrane form of this molecule. $M_r \times 10^{-3}$ are shown.

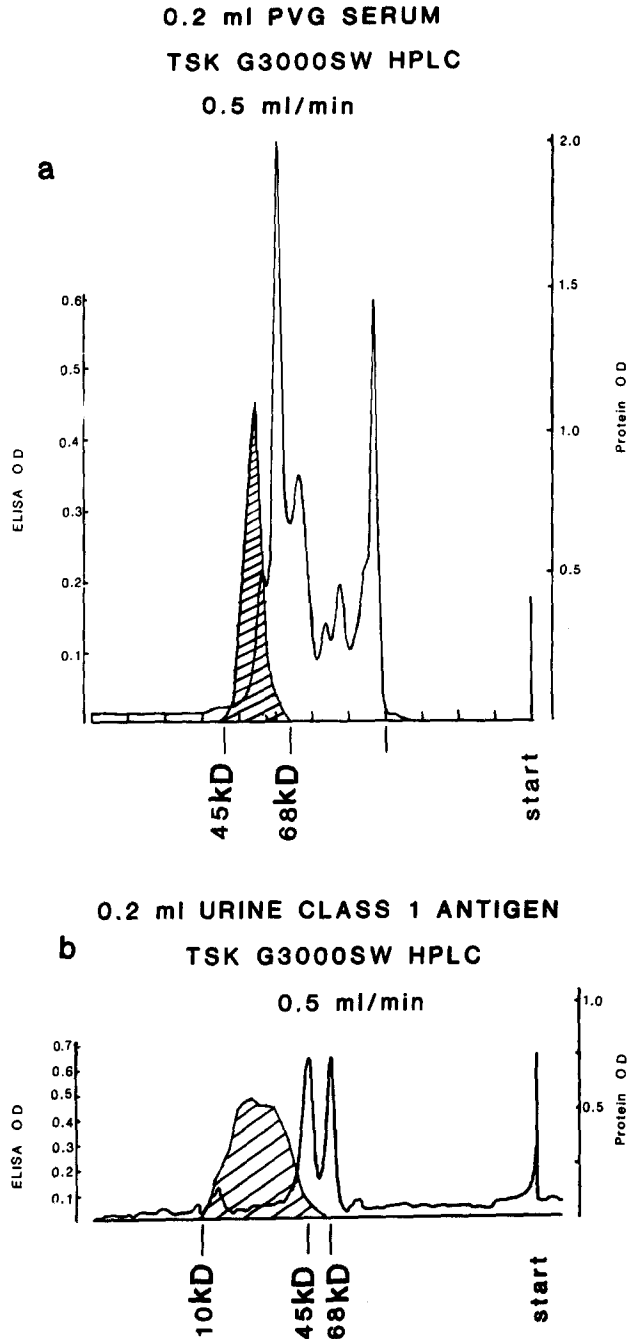


FIGURE 3. HPLC gel filtration of DA serum and urine. DA serum (200 μ l) was fractionated on an LKB TSK G3000SW column (LKB Produkter) running at 0.5 ml/min in PBS-azide. The serum RT1A^{av1} molecules, eluted as a sharp peak (shaded area) at the trailing edge of the serum protein peaks with an apparent M_r between that of BSA (68×10^3) and OVA (43×10^3), showing that the molecules exist as soluble monomers in the circulation. Class I molecules purified from the urine showed that antigenic material elutes from this column with a wide range of apparent M_r from 50×10^3 down to $\sim 15 \times 10^3$.

of soluble class I molecules by an inhibition of the binding to DA red cell targets, of ^{125}I -labeled mouse anti-RT1A mAb F16.4.4. This assay showed that soluble molecules were present in the sera of all the strains tested (Fig. 4).

Quantitation and Turnover of Serum Class I Molecules. As both erythrocytes and leu-

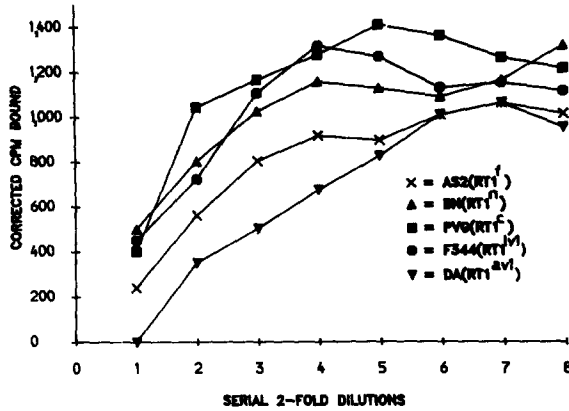


FIGURE 4. Soluble class I antigens detected in the sera of five rat strains. The mouse anti-rat F16.4.4 mAb binds to a monomorphic determinant present on the RT1.A class I molecules of all rat strains. The binding of this antibody to DA RBC was competitively inhibited by serial twofold dilutions of rat serum. All five sera were inhibitory indicating the presence of class I molecules in solution in the sera.

kocytes in the blood of normal rats also express membrane-bound class I molecules, the concentration of molecules in the cellular and liquid phases of the blood was measured. The number of molecules on the RBC surface was measured by Scatchard analysis (12) at 4,500 sites per PVG RBC, and 10,800 sites per DA RBC. These erythrocytes were then used as a calibrated source of antigen to measure the concentration of molecules in the serum by saturation analysis (13). The results (19) showed that the concentration of class I molecules is 379 ng/ml in PVG serum, and 347 ng/ml in DA serum. This is $\sim 4\%$ of the concentration of membrane-bound class I molecules on the erythrocytes in the same volume of blood (10 $\mu\text{g}/\text{ml}$) and is ~ 25 times the concentration on the leukocyte membranes in this volume (16 ng/ml). The concentration in efferent lymph draining from lymphoid tissue is about twice this level (19).

The half-life of the serum molecule was measured by capitalizing on the facts that neonatally tolerant rats contain only trace amounts of donor antigen in their circulation (19) and exhibit no reactivity against donor MHC molecules (14). Tolerant rats were injected with donor serum and the level of donor antigens in their blood was measured by calibrated ELISA at intervals. These experiments showed that the decay of donor molecule was exponential, with a half-life of 2.7 h (Fig. 5).

Cellular Origin of Soluble Class I Molecules. The origin of these soluble molecules

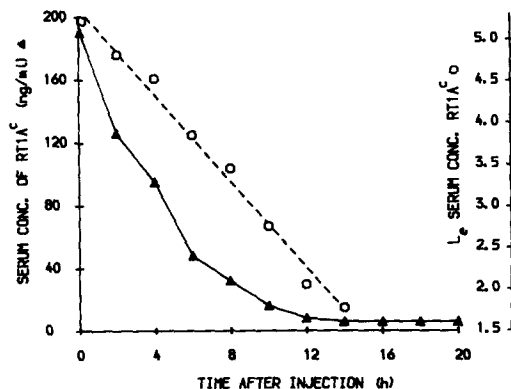


FIGURE 5. Half-life of serum class I molecules. Soluble class I molecules disappear from the recipients' blood exponentially (\blacktriangle) with a gradient (λ) on a semilogarithmic plot (\circ) of -0.2608 . From classical pharmacokinetics: $T_{1/2} [\log_e 2 / -\lambda] = 2.66$ h.

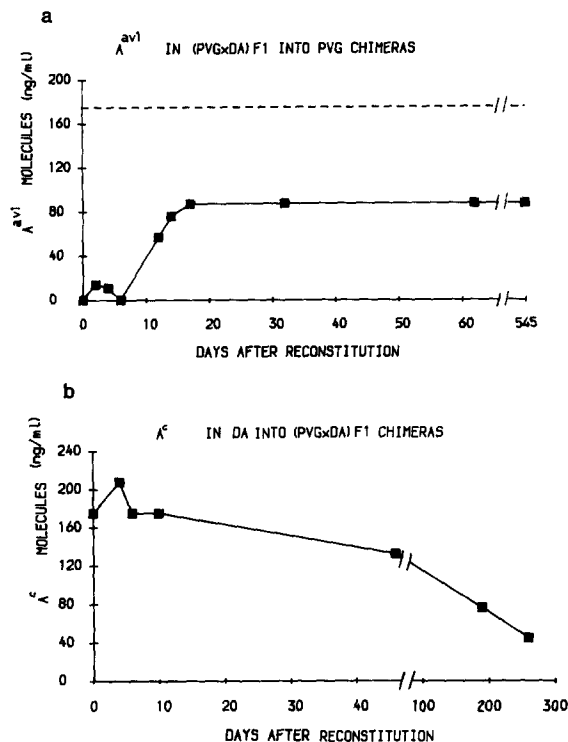


FIGURE 6. The origin of soluble class I molecules from the hemopoietic system. (a) In radiation chimeras of the (PVG \times DA)F₁ into PVG type the levels of A^{av1} molecules derived from the unshared DA parental antigen of the F₁ remain undetectable until 10 d after irradiation and injection of F₁ bone marrow. Thereafter these molecules increase rapidly in concentration reaching about half the level present in normal F₁ animals by \sim 17 d. Thereafter they remain at that level indefinitely. This experiment has also been done using (PVG \times DA)F₁ bone marrow given to DA recipients and analysis of serum of the recipient for the A^c molecule with identical results. (b) In chimeras of the DA into (PVG \times DA)F₁ type the levels of host-derived A^c molecules fall slowly over a period of several months. Even at 280 d after reconstitution, host-derived molecules are present at \sim 25% of the level found in normal F₁ animals.

was studied in radiation chimeras. In the F₁ into parent chimeras, levels of serum class I molecules derived from the F₁ unshared partner reached maximal values at 17 d after reconstitution and remained high (Fig. 6 a) showing that cells of the hemopoietic series were the source of at least half of the serum molecules. The kinetics of appearance of soluble molecules in these chimeras suggests that the secreting cell of hemopoietic origin emerges from the bone marrow in significant numbers within 2–3 wk.

In the parent into F₁ chimeras the loss of soluble molecules of unshared parental type from the circulation was slow but inexorable (Fig. 6 b) suggesting that they were produced by cells with a long tissue-residence time which were slowly lost from the body after irradiation and replaced by donor-derived cells. The fact that levels of these molecules do not fall to zero leaves open the question of whether other somatic cells also secrete soluble class I molecules into the blood.

Since the level of host-type molecules remains high for >40 d after irradiation, we can exclude radiosensitive cells such as lymphocytes as the source of these molecules. A requirement for T lymphocytes in soluble antigen production was also formally excluded by showing that the levels of A^c molecules in the circulation of mutant, athymic, nude rats of the PVG-*rnu/rnu* strain, which lack peripheral T cells, were similar to the levels in normal, euthymic PVG animals, i.e., 380 ng/ml (Fig. 7).

In vitro cultures of lymph node lymphocytes, spleen cells, and peritoneal cells were set up to ascertain whether release of soluble class I molecules could be detected in vitro. Peritoneal cells produced ~ 100 ng of class I molecules per 10^6 cells

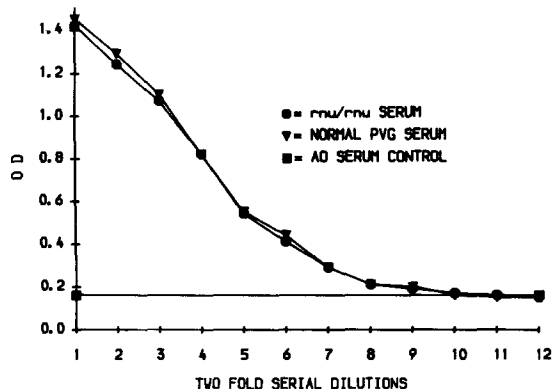


FIGURE 7. The concentrations of RT1.A^c antigen in the PVG *rnu/rnu* athymic nude rat. A comparison of the titration of normal PVG serum (▼) from a euthymic animal with that from a congenitally athymic nude rat (●) shows that the concentration of RT1.A^c molecules in the sera of these two rat strains is equivalent. Since the nude rat has no mature, peripheral T cells, these cells cannot be the source of soluble class I molecules.

per 24 h for the first 3 d of culture. Spleen cells produced about half this amount and lymph node cells produced much smaller amounts (Fig. 8). No antigen was released by erythrocytes (data not shown). The most likely source of a major portion of soluble class I molecules *in vivo* therefore appears to be a cell of the macrophage or dendritic cell lineage. This is consistent with the observation that lymph draining from lymphoid tissues rich in cells of this type contains high levels of soluble antigen (19).

Urinary Excretion of Soluble Class I Molecules. The presence of soluble class I molecules in the urine suggested that the rapid half-life could be due to excretion. Soluble class I molecules were readily detectable in the urine (Fig. 1) and calibration of the ELISA with the serum molecule showed that the concentration of class I molecules in the urine was quite variable and ranged between 40 and 190 ng/ml. Proof that the urine molecules were derived from the blood was obtained by infusing known amounts of PVG donor antigen into the blood of DA rats tolerant of PVG and detecting its excretion in the urine. We could account for only 6.5% of the injected antigen in the urine collected over the subsequent 48 h (1.9 μ g injected, 124 ng recovered).

A normal 300 g PVG rat loses from the blood \sim 18 μ g of class I molecules each

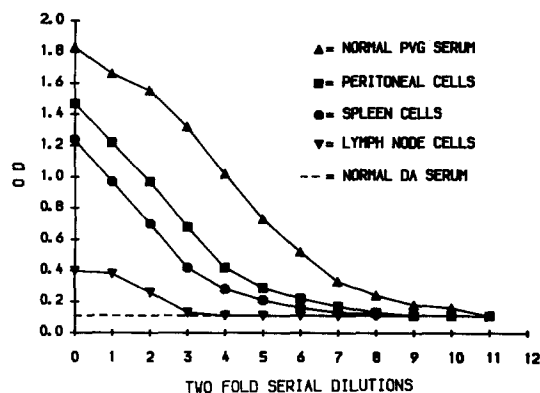


FIGURE 8. Secretion of RT1.A^{av1} class I antigen *in vitro*. Cultures of equivalent numbers (10^6 /ml) or lymph node lymphocytes, (▼) spleen cells (●) and peritoneal cells (■) *in vitro* and assay of their supernatants after 2 d of culture showed that only peritoneal cells and spleen cells produced significant concentrations of antigen compared with control normal serum (---).

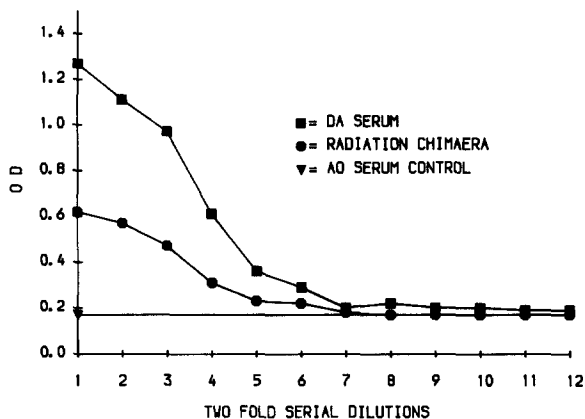


FIGURE 9. RT1.A^{av1} class I antigens in the urine of (PVG × DA)_{F1} into PVG radiation chimeras. Unshared parental class I molecules are found in the urine of _{F1} into parent radiation chimeras (●) in concentrations that vary from a quarter of that found in serum (■) to levels that are barely detectable by the ELISA.

24 h (whole blood concentration 190 ng/ml, blood volume 7% of body weight = 21 ml, half-life 2.7 h). With an average 24-h urine volume of 14 ml this gives an estimated detectable urine concentration of 83 ng/ml, a figure in reasonable agreement with the measured urine concentrations of between 40 and 190 ng/ml. Thus, only a fraction of the class I molecules normally secreted into the blood are detected by ELISA in the urine. The class I molecules purified from the urine by affinity chromatography on mAb columns not only have the typical 39-kD heavy chain and 13-kD light chain associated with soluble class I molecules, but also show a major protein band at 30 kD (Fig. 2), suggesting that there is fragmentation of the class I molecules occurring in the urine.

The actual degree to which these molecules are degraded is not revealed by SDS-PAGE analysis, as very small fragments would run off the bottom of the gel. In addition, the cleavage products might be present in a variety of sizes and would not show as localized bands on the gel. We therefore fractionated class I antigen, which had been extracted from the urine, by gel-filtration HPLC and assayed the fractions by ELISA (Fig. 3 *b*). This showed that antigenic material emerges from the HPLC column as a broad band of antigenic activity from ~ 50 down to $15 \times 10^3 M_r$. This is in sharp contrast to the behavior of the class I molecules in serum, which emerge from the HPLC column as a well-defined narrow peak between 68 and $43 \times 10^3 M_r$ (Fig. 3 *a*). Since only those fragments carrying two spatially separate epitopes are detected by ELISA, it is probable that a major population of smaller degraded fragments is also present and escapes detection. This could explain the low yield of foreign class I molecules detected in the urine after intravenous injection.

An alternative explanation would be that most of the class I molecules lost from the blood are removed by some other mechanism and only a minority are excreted in the urine. If excretion via the kidneys is a major pathway, interruption of renal excretion should cause an increase in blood levels of class I molecules in proportion to the fraction of molecules which are removed from the blood by the kidneys. 6 h after bilateral nephrectomy of four PVG rats, the mean serum level of class I A^c molecules was measured to be 866 ng/ml by calibrated ELISA. The calculated serum level, assuming that all of the serum molecules are normally excreted in the urine, is 806 ng/ml. Renal excretion is therefore the major if not the only fate of

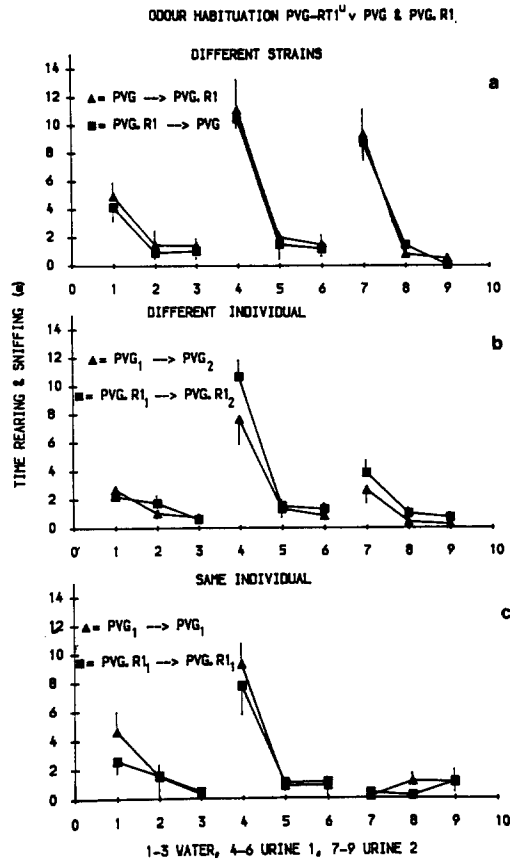


FIGURE 10. Mean time (\pm SEM) in seconds spent rearing and sniffing at urine of PVG or PVG.R1 donors on each 2 min presentation by PVG-RTI^U subjects. Water was presented to the subjects on trials one to three for all three experiments. Urine 1 (trials four to six) corresponds to the urine donor that precedes the arrow in each panel and urine 2 (trials seven to nine) the urine donor that follows it.

the class I molecules in solution in the blood. Once in the urine the majority of these molecules undergo rapid degradation to small fragments.

In radiation chimeras of the F₁ into parent type, soluble class I molecules derived from the unshared haplotype of the F₁, which were present in high concentrations in the blood (Fig. 6 a) of the donor, were also readily detectable in the urine (Fig. 9).

The excretion of class I antigens in the urine has also been reported in man, although this has apparently only been studied in patients with renal pathology (20), including renal transplant recipients. In these patients the class I molecules were shown to be of recipient type and did not carry the allelic specificities of the kidney donor (21).

Olfactory Detection of MHC Class I Associated Urinary Odors. The presence of classical class I transplantation antigens of the A^{av1} and A^c type and their degradation products in the urine of normal rats prompted us to see whether rats could also detect urinary odors associated with these molecules. In these experiments we used the habituation-dishabituation method (16) in which untrained detector animals were habituated to the odor of urine samples from donors of one strain and then tested for dishabituation when exposed to urine from donors of the other strain. To exclude a role for any variable other than the MHC class I molecules in these experiments,

throughout we used male rats from the PVG congenic series (22). Fig. 10 *a* shows that the PVG-RT1^u rats could readily discriminate between the odor of PVG urine containing the A^c molecule and PVG.R1 urine containing the A^{av1} molecule. 20 males were tested, 10 in the order PVG then PVG.R1 and 10 in the reverse order (PVG.R1 then PVG). There were significant differences in time spent rearing and investigating odors over the nine tests for both groups (PVG then PVG.R1, $F = 14.89$; $df = 8, 72$; $p < 0.001$. PVG.R1 then PVG, $F = 16.24$, $df = 8, 72$; $p < 0.001$). Post-hoc Newman-Keuls tests indicated that for both groups of subjects, more time was spent investigating odors on trials 4 and 7 than on any other odor presentation ($p < 0.01$).

Fig. 10 *b* shows the time spent investigating urine from two individuals of the same strain. The 14 subjects tested with urine from two male PVG rats (PVG₁ then PVG₂) showed significant differences in investigation time over the nine odor presentations ($F = 11.03$; $df = 8, 104$; $p < 0.001$) and more time was spent investigating the odor on trial four than on any other trial ($p < 0.01$). The time investigating the odor on trial seven did not differ from that on trial five or six, indicating that the odors from two different PVG males were not discriminated.

The 20 subjects tested with urine from two PVG.R1 males (PVG.R1₁ then PVG.R1₂) also showed significant differences in investigation time over the nine odor presentations ($F = 37.42$; $df = 8, 152$; $p < 0.001$), and more time was spent investigating odors on trials four and seven than on any other trials ($p < 0.01$). The increase in odor investigation on trial seven indicates that the subjects could discriminate between two individual PVG.R1 males by their urine odor.

Fig. 10 *c* shows the responses of subjects to two urine samples from the same individual. The eight subjects tested with separate urine samples from PVG males (PVG₁ then PVG₁) showed a significant difference in investigation times over the nine trials ($F = 15.30$; $df = 8, 56$; $p < 0.001$) and investigated the odor on trial four more than on any other trial. Similar results were obtained for the eight males tested with separate urine samples from individual PVG.R1 males (PVG.R1₁ then PVG.R1₁) ($F = 7.02$; $df = 8, 56$; $p < 0.001$), with more investigation during trial four than any other trial ($p < 0.01$).

Discussion

Classical class I transplantation antigens have mostly been studied as membrane bound molecules that participate in graft rejection and antigen presentation (1). There are, however previous reports of soluble forms of these molecules in true solution in the body fluids of mouse (23, 24), man (20, 25) and rat (18, 26).

The present study confirms that such molecules exist in the rat, and that ~50% of them are secreted into the blood by cells of the hemopoietic system (Fig. 6, *a* and *b*). The mechanism of secretion is unknown. Conversion of a typical membrane-anchored molecule into a secreted form could occur at the DNA, RNA, or protein level. At the DNA level, class I genes comprise eight exons, which correlate well with the structural and functional domains of the molecule (27). Deletion or modification of exon 5 (which encodes the transmembrane region) could lead to a secreted product. Such a mechanism has been postulated for the secretion of a non-polymorphic class I polypeptide, which is detectable in mouse serum (28, 29). This

molecule is secreted because exon 5 contains numerous substitutions, a frameshift, and a termination codon, resulting in a truncated polypeptide lacking a true hydrophobic region.

At the RNA level, alternate splicing of RNA to delete the hydrophobic coding region is well known as the mechanism of switching between membrane-bound and secreted Ig. A similar process has recently been shown to result in switching from membrane insertion to secretion of the classical class I HLA-A2 antigens in vitro (30, 31).

The truncated heavy chain of the soluble class I molecules is consistent with either of the above two mechanisms, but is also consistent with proteolytic cleavage of class I molecules from cell membranes at or near the juxta-membranous papain cleavage site.

Quantitation of the serum molecule (19) has shown it to be in the range of 350 to 380 ng/ml in sera of normal animals.

From previous experiments, the rate of secretion of soluble class I molecules into the circulation was anticipated as being high because liver-grafted rats showed near-maximal levels of donor antigen in their circulation within 24 h of operation, (32, 33). Accordingly, the half-life was measured and found to be 2.7 h. Assay of the urine showed that the serum molecule was constitutively excreted via the kidneys in the urine where >90% of the molecules underwent degradation (Figs. 2 and 3 *b*).

The excretion of classical class I molecules in the urine seemed to us important since olfactory discrimination in both mice and rats has been shown to include the ability to identify urine samples from congenic animals that differ only at the MHC (34, 35). Also, up to 90% of pregnant female mice will abort their preimplantation embryos when exposed to the odor of urine from a foreign male (36) even when the male is from an inbred strain congenic with that of the original stud and differs only at the MHC (37) or is a mutant (*bm1*) at one class I MHC locus (38). The only known difference between *bm1* and the C5BL/6 wild type are point mutations in the class I gene resulting in MHC molecules that differ by three amino acids. This implies that the olfactory cues in the urine are derived not from the products of loci closely linked to class I, but either directly or indirectly from the product of the class I genes themselves.

The results obtained from the behavioral experiments described in this paper indicate that the urine odors of male rats of the PVG and PVG.R1 strains are equally attractive, as they are both investigated to the same degree on trial four. PVG and PVG.R1 individuals are readily discriminated as evidenced by the dishabituation on trial seven (Fig. 10 *a*). Individual PVG males are not discriminable by their urine odors, but PVG.R1 males appear to be. This latter result was unexpected since this is a congenic recombinant inbred strain where each rat is thought to be genetically identical. However, since PVG.R1 is at the 12th generation of backcross there is a possibility that residual heterozygosity at some background loci may contribute to variation in the odors of individuals within this particular strain.

Behavioral studies of this type, which revealed such intimate linkage between MHC class I genotype and urinary odor, have previously been interpreted as reflecting the action of immune response gene effects of the class I genetic locus (39), possibly controlling the host immune responses against commensal bacterial flora of the skin, urinary tract, or the gut. Individual MHC haplotypes would then be associated with unique flora. The volatile odorants in the excretions were thought to be secondary

metabolites derived from these organisms. However, it was recently found that in radiation chimeras of the F_1 into parent type, urinary odors are of F_1 type (40). Since it has been shown that class I-restricted immune responsiveness is heavily biased towards the parental MHC (3, 4), it would be expected that class I-associated immune response phenotype would remain parental in these animals. Urinary odors therefore do not show correlation with putative class I immune response gene effects but do show close correlation with the soluble class I molecules themselves described in this study (Fig. 9). In addition, Yamazaki et al. (41) have shown that urine collected from mice that are congenic with respect to the nonclassical Qa , Tla class I loci can be distinguished by odor alone. These molecules have no known Ir gene function. They are, however, found in the circulation (42, 43).

Class I molecules themselves would seem to be unlikely candidates as the odoriferous component in the urine (44). There are two possible ways in which the excretion of these molecules might confer the class I-associated odor. First, the odoriferous components of the urine could be volatile fragments of the MHC molecules. This mixture of small fragments, which would of course be unique to the particular polymorphic class I molecules excreted, may be the specific odorants. Interestingly, rats that are able to discriminate the PVG and PVG.R1 strains by smelling their urine that contains the A^c and A^{av1} molecules in both degraded and intact forms (Figs. 2 and 3 *b*), fail to distinguish between these strains by the smell of their serum (44) that contains the molecules only in the intact form (Figs. 2 and 3 *a*).

Second, MHC glycoproteins are well known as associative molecules. It is possible that the ability of MHC class I molecules to associate in a selective way with other small molecules could also be the mechanism by which a unique mixture of endogenous volatile metabolites is transported by class I MHC glycoproteins from the blood into the urine. This mixture, although derived from a similar metabolic pool in each individual (as shown by the attractive but nonunique odor of sera from different strains) (44), would be special to the particular class I molecules involved in its transport and would therefore impart a unique odor to the urine. The volatile molecules we postulate have not yet been identified, but strong candidates may be the small volatile components identified by Schwende et al. (45) as being present in mixture patterns specific to particular MHC haplotypes in the urine of congenic mice. The notion of a carrier that binds smaller volatile molecules is not new and has been shown for two primer pheromone effects (46, 47). There are several suggested advantages of such a scheme: the slow, steady release of small volatiles from the carrier; control of excretion of the volatile molecules; and the protection of these volatiles from decomposition.

Olfactory cues, provided by MHC-specific molecules in the urine, may be used in mate selection. The trend is to choose mates that are different at the MHC (35). This trend could force the observed high levels of MHC heterozygosity in breeding demes (48) and is so powerful, at least in the mouse, that an established pregnancy is aborted if the opportunity for more extensive outbreeding is provided (36, 37, 49). Since recombination due to crossing over within the MHC is common, repeated cycles of mate selection for foreign MHC alleles plus genetic recombination and mutation ensure extensive polymorphism of alleles and haplotypes within species. Thus the MHC class I molecules could act as individuality markers, used by breeding animals in the wild to provide the information on which their strategy for outbreeding is based.

Summary

Classical class I transplantation antigens present in solution in the body fluids have been studied. These antigens have been found in a monomeric, soluble form in blood, lymph, and urine, and a major source is the hemopoetic system which gives rise to cells that secrete these molecules into the blood. The cell types most probably involved in their secretion are of the macrophage/dendritic cell lineage. The serum molecule is a heterodimer with a heavy chain of 39,000 mol wt associated noncovalently with β_2 -microglobulin and is present in serum at a concentration between 350 and 390 ng/ml. These molecules have a short half-life of 2.7 h and are excreted into the environment via the kidneys in the urine. In the urine, >90% of the molecules are degraded into smaller fragments.

This finding that normal metabolic processes lead to the excretion of classical highly polymorphic class I molecules in the urine provides a direct explanation in molecular terms of the ability of animals to identify individuals on the basis of urinary odor. Since intact class I molecules are unlikely to be the odoriferous component in the urine, two hypotheses have been suggested. Either small fragments of class I molecules are detected or the molecule acts as a carrier that transports volatiles from the serum into the urine where they are released, giving rise to the class I-associated odor.

We are grateful to E. A. Boyse, G. W. Butcher and J. C. Howard for comments on the manuscript, to E. C. Bate-Smith and R. van Aarde for useful discussions and particularly to A. Diamond, J. C. Howard and G. W. Butcher for generous gifts of mAbs and advice on their use.

Received for publication 9 December 1987 and in revised form 29 February 1988.

References

1. Klein, J. 1986. *The Natural History of the Major Histocompatibility Complex*. John Wiley & Sons, New York.
2. Duncan, W. R., E. K. Wakeland, and J. Klein. 1979. Histocompatibility-2 system in wild mice. VIII. Frequencies of H-2 and Ia antigens in wild mice from Texas. *Immunogenetics*. 9:261.
3. Bevan, M. J., and P. J. Fink. 1978. The influence of thymus H-2 antigens on the specificity of maturing killer and helper cells. *Immunol. Rev.* 42:3.
4. Zinkernagel, R. M., and P. C. Doherty. 1979. MHC-restricted cytotoxic T cells: studies on the biological role of polymorphic major transplantation antigens determining T-cell restriction-specificity, function, and responsiveness. *Adv. Immunol.* 27:51.
5. Klein, J. 1979. The major histocompatibility complex of the mouse. *Science (Wash. DC)*. 303:516.
6. Diamond, A. G., A. P. Larkins, B. Wright, S. T. Ellis, G. W. Butcher, and J. C. Howard. 1984. The alloantigenic organization of RT1A^a, a class I major histocompatibility complex molecule of the rat. *Eur. J. Immunol.* 14:405.
7. Stephenson, S. P., A. M. Livingstone, J. C. Howard, and G. W. Butcher. 1985. The specificity of responses against RT1.C antigens. *Transplant. Proc.* 17:1844.
8. Parham, P. 1979. Purification of immunologically active HLA-A and -B antigens by a series of monoclonal antibody columns. *J. Biol. Chem.* 254:8709.
9. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680.
10. Hart, D. H. J., and J. W. Fabre. 1981. Problems in the use of erythrocytes for RT1.A

- typing studies, probably due to quantitative differences in RT1.A antigen expression in different strains. *Transplant. Proc.* 13:1329.
11. Heusser, C., M. Boesman, J. H. Nordin, and H. Isliker. 1973. Effect of chemical and enzymatic radioiodination on in vitro human C1q activities. *J. Immunol.* 110:820.
 12. Scatchard, G. 1949. The attractions of proteins for small molecules and ions. *Ann. NY Acad. Sci.* 51:660.
 13. Ekins, R. P. 1974. Basic principles and theory. *Brit. Med. Bull.* 30:3.
 14. Roser, B., and S. E. Dorsch. 1979. The cellular basis of transplantation tolerance in the rat. *Immunol. Rev.* 46:55.
 15. Ford, W. L., W. Burr, and M. Simonsen. 1970. A lymph node weight assay for the graft-versus-host activity of rat lymphoid cells. *Transplantation (Baltimore).* 10:258.
 16. Sundberg, H., K. Doving, S. Novikov, and H. Ursin. 1982. A method for studying responses and habituation to odors in rats. *Behav. Neural Biol.* 34:113.
 17. Kirk, R. E. 1968. *Experimental Design: Procedures for the Behavioral Sciences.* Brooks/Cole Publishing Co., Belmont, CA. 000 pp.
 18. Zimmermann, F. A., G. W. Butcher, H. S. Davies, G. Brons, N. Kamada, and O. Turel. 1979. Techniques for orthotopic liver transplantation in the rat and some studies of the immunologic responses to fully allogeneic liver grafts. *Transplant. Proc.* 11:571.
 19. Singh, P. B., R. E. Brown, and B. Roser. 1987. MHC antigens in urine as olfactory recognition cues. *Nature (Lond.).* 327:161.
 20. Vincent, C., and J. P. Revillard. 1979. Characterization of molecules bearing HLA determinants in serum and urine. *Transplant. Proc.* 11:1301.
 21. Robert, M., C. Vincent, and J. P. Revillard. 1974. Presence of HL-A antigens and β_2 -microglobulin in tubular proteinuria. *Transplantation (Baltimore).* 18:89.
 22. Butcher, G. W., and J. C. Howard. 1986. The MHC of the laboratory rat, *Rattus norvegicus*. In *Handbook of Experimental Immunology*, 4th ed. Ed. D. M. Weir. Blackwell Scientific Publications, Oxford. Section 101.
 23. Callahan, G. N., S. Ferrone, J. P. Allison, and R. A. Reisfeld. 1975. Detection of H-2 antigens in serum. *Transplantation (Baltimore).* 20:431.
 24. Callahan, G. N., S. Ferrone, M. D. Poulik, R. A. Reisfeld, and J. Klein. 1976. Characterization of Ia antigens in mouse serum. *J. Immunol.* 117:1351.
 25. Van Rood, J. J., A. Van Leeuwen, and M. C. T. Van Santen. 1970. Anti HL-A2 inhibitor in normal human serum. *Nature.* 226:366.
 26. Spencer, S. C., and J. W. Fabre. 1987. Water-soluble form of RT1.A class I MHC molecules in the kidney and liver of the rat. *Immunogenetics.* 25:91.
 27. Hood, L., M. Steinmetz, and B. Malissen. 1973. Genes of the Major Histocompatibility Complex of the mouse. *Annu. Rev. Immunol.* 1:529.
 28. Cosman, D., G. Khoury, and G. Jay. 1982. Three classes of mouse H-2 messenger RNA distinguished by analysis of cDNA clones. *Nature (Lond.).* 295:73.
 29. Cosman, D., M. Kress, G. Khoury, and G. Jay. 1982. Tissue-specific expression of an unusual H-2 (class I)-related gene. *Proc. Natl. Acad. Sci. USA.* 79:4947.
 30. Krangel, M. S. 1985. Unusual RNA splicing generates a secreted form of HLA-A2 in a mutagenized B lymphoblastoid cell line. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:1205.
 31. Krangel, M. S. 1986. Secretion of HLA-A and -B antigens via an alternative RNA splicing pathway. *J. Exp. Med.* 163:1173.
 32. Roser, B. J., N. Kamada, F. Zimmermann, and H. ff. S. Davies. 1983. Immunosuppressive effect of experimental liver allografts. In *Liver Transplantation.* Ed. R. Y. Calne. Grune and Stratton, London. p 35-54.
 33. Kamada, N., H. ff. S. Davies, and B. Roser. 1981. Reversal of transplantation immunity by liver grafting. *Nature (Lond.).* 292:840.
 34. Beauchamp, G. K., K. Yamazaki, and E. A. Boyse. 1985. The chemosensory recognition of genetic individuality. *Sci. Am.* 253:86.

35. Yamazaki, K., E. A. Boyse, V. Miké, H. T. Thaler, B. J. Mathieson, J. Abbott, J. Boyse, Z. A. Zayas, and L. Thomas. 1976. Control of mating preferences in mice by genes in the major histocompatibility complex. *J. Exp. Med.* 144:1324.
36. Dominic, C. J. 1966. Observations on the reproductive pheromones of mice. I. Source. *J. Reprod. Fertil.* 11:407.
37. Yamazaki, K., G. K., Beauchamp, C. J. Wysocki, J. Bard, L. Thomas, and E. A. Boyse. 1983. Recognition of H-2 types in relation to the blocking of pregnancy in mice. *Science (Wash. DC)*. 221:186.
38. Yamazaki, K., G. K. Beauchamp, O. Matsuzaki, D. Kupriewski, J. Bard, L. Thomas, and E. A. Boyse. 1986. Influence of a genetic difference confined to mutation of H-2K on the incidence of pregnancy block in mice. *Proc. Natl. Sci. USA*. 83:740.
39. Howard, J. C. 1977. H-2 and mating preferences. *Nature (Lond.)*. 266:406.
40. Yamazaki, K., G. K. Beauchamp, L. Thomas, and E. A. Boyse. 1985. The hematopoietic system is a source of odorants that distinguish major histocompatibility types. *J. Exp. Med.* 162:1377.
41. Yamazaki, K., G. K. Beauchamp, J. Bard, L. Thomas, and E. A. Boyse. 1982. Chemosensory recognition of phenotypes determined by the *T1a* and *H-2K* regions of chromosome 17 of the mouse. *Proc. Natl. Acad. Sci. USA*. 79:7828.
42. Maloy, W. L., J. E. Coligan, Y. Barra, and G. Jay. 1984. Detection of a secreted form of the murine H-2 class I antigen with an antibody against its predicted carboxyl terminus. *Proc. Natl. Acad. Sci. USA*. 81:1216.
43. Kress, M., D. Cosman, A. Khoury, and G. Jay. 1983. Secretion of a transplantation-related antigen. *Cell*. 34:189.
44. Brown, R. E., P. B. Singh, and B. J. Roser. 1987. The major histocompatibility complex and the chemosensory recognition of individuality in rats. *Physiol. & Behav.* 40:65.
45. Schwende, F. J., J. W. Jorgenson, and M. Novotny. 1984. Possible chemical basis for histocompatibility-related mating preference in mice. *J. Chem. Ecol.* 10:1603.
46. Marchlewska-Koj, A. 1977. Pregnancy block elicited by urinary proteins of male mice. *Biol. Reprod.* 17:729.
47. Vandenberg, J. G., J. S. Finlayson, W. J. Dobrogosz, S. S. Dills, and T. A. Kost. 1976. Chromatographic separation of puberty accelerating pheromone from male mouse urine. *Biol. Reprod.* 15:260.
48. Duncan, W. R., E. K. Wakeland, and J. Klein. 1980. Heterozygosity of *H-2* loci in wild mice. *Nature (Lond.)*. 281:603.
49. Bruce, H. M. 1959. An exteroceptive block to pregnancy in the mouse. *Nature (Lond.)*. 184:105.