*Immunogenetics* 7:185–200, 1978

*Immunogenetics*
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**Original Investigations**

# An Ly-Like Specificity with Extensive Nonlymphoid Expression

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**Abstract.** The characteristics of a strong mouse alloantigen with renal, bone marrow, and lymphoid expression were studied. This antigen is probably identical to that currently designated Ly-6.2. It was defined by the high-titered (1 : 1000) cytotoxic activity of three different antisera against peripheral lymphocyte target cells from DBA/2, DBA/1, and a variety of other strains. In the $F_2$ and four backcross generations the genetic control of this specificity segregated as a single autosomal dominant gene. In lymphoid tissues the predominant expression was on T cells but 10–30% of B cells were lysed by these antisera. The specificity was expressed strongly in kidney, as shown by sequential absorption, in amounts equal to or greater than the amount in lymphoid tissues. Comparison to the rate of absorption of H-2 by kidney indicated that this antigen may be expressed in amounts comparable to an H-2 antigen in kidney. Immunization with kidney tissue resulted in a strong cytotoxic antibody response. The number of bone marrow cells expressing this antigen (40–50%) was well beyond what could be accounted for by T lymphocytes in bone marrow. In addition, a nonlymphoid tumor, the P815Y mastocytoma, was positive by cytotoxicity and by absorption. The extensive nonlymphoid expression and antigenic strength of Ly-6.2 raises the possibility that this serologically defined lymphocyte alloantigen will have histocompatibility effects when allografts of the appropriate tissues are examined.

## Introduction

Serologically detected mouse alloantigens controlled by genes remote from *H-2* vary widely in their tissue distribution. Characteristic patterns of expression in various tissues thus form a rational basis for the classification of these antigens into groups with common characteristics (Snell *et al.* 1976, Boyse *et al.* 1977). One such group is comprised of the erythrocyte or Ea antigens; another group is the lymphocyte or Ly antigens, characterized by their exclusive expression in lymphoid tissues. A heterogeneous third group is named for some distinctive characteristic of their tissue expression, for example, the Thy-1 and PC-1 antigens. This group is distinguished from the Ly antigens by their prominent expression in nonlymphoid tissue, such as brain, skin, etc. A recently described alloantigen, Ly-6.2, is unusual among the Ly

0093–7711/78/0007/0185/$03.20

antigens in that as well as being expressed on T lymphocytes, it has some expression in kidney by absorption (McKenzie *et al.* 1977). This antigen seems to hold an equivocal position between the Ly antigens, which typically have no nonlymphoid expression, and the antigens of the third group, which have both lymphoid and nonlymphoid expression.

The present study focuses on a non-H-2 specificity which is probably identical to Ly-6.2. Unlike the original antiserum defining Ly-6.2, which is raised in a strain combination requiring a recombinant inbred line which is not generally available, our antisera defining this specificity were made in three strain combinations which are commercially available, and thus permit wider study of this specificity. We have also found that this specificity is expressed surprisingly strongly in kidney (as strongly as in lymphoid tissues) and is immunogenic in kidney. Furthermore, it is expressed extensively on nonlymphoid bone marrow cells and on a nonlymphoid tumor, and is found on some B and T lymphocytes. Taken together, these findings suggest that the specificity currently designated Ly-6.2 is in fact a unique specificity with important and unusual features in its nonlymphoid tissue distribution distinguishing it from the more typical Ly specificities, which are confined to the lymphoid system.

## Materials and Methods

*Animals.* Six to twelve week old female mice of strains A/J, AKR/J, BALB/cJ, CBA/J, C57BL6J, C57BL/10Sn (abbreviation 'B10') and its congenic partner strains B10.A/SgSn, B10.D2/nSn, and B10.A(5R)/SgSn, C57BR/cdJ, C3H/HeJ, DBA/1J, DBA/2J, NZB/BINJ, HTG/J, RF/J, SJL/J, SWR/J, and (BALB(c × A)$F_1$ were obtained from The Jackson Laboratory, Bar Harbor, Maine. In addition (BALB/c × CBA)$F_1$ and (B10 × C3H.OH)$F_1$ were bred in our animal colony from breeding animals obtained from The Jackson Laboratory or from Dr. C. S. David.

*Tumor Cells.* P815Y mastocytoma tumor cells were obtained from Dr. R. Gorczinski at the Ontario Cancer Institute and were passaged serially in the peritoneal cavities of DBA/2 mice.

*Media.* Eagles minimal essential medium (MEM) buffered with 0.026 M sodium bicarbonate was used for these assays, usually with 10% fetal calf serum (Flow Laboratories, Rockville, Maryland). For some purposes, MEM buffered with 0.02 M Hepes was used, as indicated.

*Complement.* Lyophilized rabbit serum (batches 33/11 and 33/14) was obtained from the Buxted Rabbit Co., Surrey, England, and was preabsorbed with mouse spleens to remove toxicity for mouse cells (Boyse *et al.* 1970).

*Antisera.* Hyperimmune antisera were produced by immunizing mice i.p. with mouse spleen or kidney by six injections at weekly intervals. Tissue from one animal was used to immunize ten animals. The mice were then bled from the retro-orbital plexus. Further batches of sera were produced by boosting the animals with another injection and bleeding 1 week later. Sera raised to estimate the immunogenicity of the kidney compared to spleen were produced by bleeding 1 week after the first immunization, after which the animals were rested for 2 days, then boosted and bled periodically.

All antisera utilized in these studies are listed in Table 1. Antisera B-1 and B-9 were prepared in collaboration with Dr. T. L. Delovitch at the Best Institute, Toronto. D-31 was supplied by the Transplantation Immunology Division, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, as described in the NIH catalogue and its supplements.

*Preparation of Cell Suspensions.* Spleen and lymph nodes were minced, crushed with a vinyl stopper, and filtered through gauze to remove clumps. Bone marrow cells (BMC) were prepared by irrigating the femurs and tibias with Hepes-buffered MEM with 10% fetal calf serum.

*Complement-Dependent Lysis Testing.* The $^{51}$Cr release assay (Sanderson 1964, Wigzell 1965) was performed with target cells labeled by incubating 1 × $10^7$ cells at room temperature with 100 $\mu$c of $^{51}$Cr

**Table 1.** Alloantisera Used

| Serum Designation | Strain Combination | Principal Specificity | Other Known or Potential Specificities |
|---|---|---|---|
| A8 | (B10 × C3H.OH)$F_1$ anti-B10.A(5R) | H-2D$^d$ | (I-J$^k$, I-E$^k$, Tla)[a] |
| A9 | (BALB/c × A)$F_1$ anti-DBA/2 | Ly-6.2 | Ly-1, Ly-2.1 |
| A12 | (BALB/c × A)$F_1$ anti-B10.A | Ly-6.2 | Ly-4.2 |
| A22 | (BALB/c × SWR)$F_1$ anti-B10.D2 | Ly-4.2 | |
| A24 | (BALB/c × CBA)$F_1$ anti-DBA/2 | Ly-6.2 | |
| A17K, A25K[b] | (BALB/c × A)$F_1$ anti-B10.A | Ly-6.2 | unknown |
| B-1 | A.TH anti-A.TL | Ia$^k$ | |
| B-9 | (PL/J × B6.PL-*Thy-1*$^a$)$F_1$ anti-C57BL/6 | Thy-1.2 | |
| D-31[c] | (B10 × A)$F_1$ anti-B10.D2 | H-2K$^d$ | I$^d$ |

[a] The cytotoxic activity of this serum against H-2$^d$ peripheral lymphocytes as tested in this report seems to be due exclusively to anti-H-2D$^d$

[b] These sera were raised by immunizations with kidney tissue (see Figure 3); all other sera were raised by conventional immunizations with spleen cells

[c] N.I.H. catalogue designation

sodium chromate (Amersham-Searle, Arlington Heights, Illinois) for 2 hours before washing. To each well of a Cooke V-bottomed microtiter plate (Cooke Engineering Co., Alexandria, Virginia) were added 20 μl of labeled target cells ($2 \times 10^4$) and 20 μl antiserum at appropriate dilution. The plates were incubated for 15 minutes at 37° C, appropriately diluted rabbit complement was added, and the plates incubated for a further 90 minutes at room temperature.

The microcytotoxicity assay, adapted from those previously described (Amos *et al.* 1969, Murphy and Shreffler 1975), was performed in disposable Micro-Test tissue culture plates (Falcon, Oxnard, California). Two μl of medium containing $2 \times 10^3$ cells were added to 2 μl of appropriately diluted serum, the mixture was incubated at 37° C for 30 minutes, and 2 μl diluted rabbit complement were added. The plates were then incubated for 60 minutes at room temperature, after which they were flooded with 3.75% buffered formalin. Live and dead cells were enumerated by phase-contrast microscopy. All assays were performed in triplicate and the means and standard deviations were calculated.

*Absorptions*. Tissues for absorption were collected into Hepes-buffered MEM. The tissues were finely minced and then homogenized with a vinyl stopper. The resultant cell suspension was filtered through gauze, washed, and the packed tissue was used for the absorptions. Perfusion of kidneys to remove blood (Davies and Butcher 1978) was not necessary, since heparinized DBA/2 blood was completely unable to absorb the activity in A12, even in amounts vastly in excess of what the content of blood in DBA/2 kidney was calculated to be. With single absorptions, equal volumes (usually 50 μl) of packed tissues were used to absorb an equal volume of diluted serum (1:10–1:30 as appropriate). The absorption was continued for 1 hour at room temperature on a rocking platform. Serum was recovered by centrifuging at 715 *g* for 3 minutes, then at 10,000 *g* for 5 minutes in Beckman microfuge B (Beckman Instruments, Palo Alto, California).

For sequential absorptions, the tissues were prepared as described and divided into aliquots of 20 μl packed volume for the first absorption, 15 μl for the second and third, and 10 μl for the final absorption. Two-hundred μl of diluted serum (1:10–1:20) was added to the first tube, absorbed as above, then recovered by centrifuging at 715 *g* for 3 minutes. A 30 μl sample of supernatant was removed for testing, and the remainder (170 μl) of the serum transferred for a second absorption. The process was repeated for each absorption, so that the ratio of serum to absorbing tissue was approximately constant for each of the four absorptions (10:1, 11:1, 9:1, 10:1). All serum samples were spun at 10,000 *g* for 5 minutes in Beckman microfuge B before testing against DBA/2 lymph node cells (LNC) in the $^{51}Cr$ release assay.

*The Double-Fluorescence Microcytotoxicity Assay*. This was adapted from an assay for human B-cell antigens (Van Rood *et al.* 1976). Washed LNC ($6 \times 10^6$) were suspended in 0.5 ml of phosphate-buffered saline containing 1% bovine serum albumin (PBS-BSA) and incubated with 50 μl of fluorescein-conjugated rabbit antimouse immunoglobulin (Daymar Laboratories, Willowdale, Ontario) diluted 1:3 for 5 minutes at 37° C. The anti-immunoglobulin gave good capping during this 37° C incubation, and was not cytotoxic during the subsequent incubation with complement. The cells were

then washed twice with 1% PBS-BSA and adjusted to 8 × $10^2$/ml. A sample (2 μl) of the allo-antiserum to be tested was then incubated with 2 μl of cells in a tissue typing plate (Searle Diagnostics, England) under paraffin oil for 30 minutes at 37° C. Then 2 μl of appropriately diluted rabbit complement were added and the plate was incubated for 1 hour at 25° C. Finally 1 μl of 1 : 70 dilution of ethidium bromide (Sigma Chemical Co., St. Louis, Missouri) dissolved in 5% EDTA in saline was added. After draining the paraffin oil and putting a cover glass on the plate, the cells were observed on an epifluorescence Leitz Orthoplan microscope using a 4BG38 filter. The B cells (usually 32–38% of the total cells) and non-B cells could be differentiated by the green cap on the former, while the dead cells were stained red by the ethidium bromide. The background kill was usually only 5%. The percentage of B cells and of non-B cells lysed by each antiserum was determined. Test and control antisera were coded to prevent observer bias.

## Results

### *Production of Antisera Against Specificity Ly-6.2*

The initial serum defining Ly-6.2 was raised in a strain combination (BALB/c × A)$F_1$ anti CXBD (Mackenzie *et al.* 1977). Because strain CXBD is a recombinant inbred line and is not widely available, we did not use this serum for the present study. However, several antisera were produced with commercially available strains which reacted strongly with a single specificity very similar to that described under the name Ly-6.2. The first of these sera (BALB/c × A)$F_1$ anti B10.A (designated A12), was raised by us in an attempt to produce a serum against Ly-4.2. However, A12 was found in microcytotoxicity to kill approximately 60% of lymph node

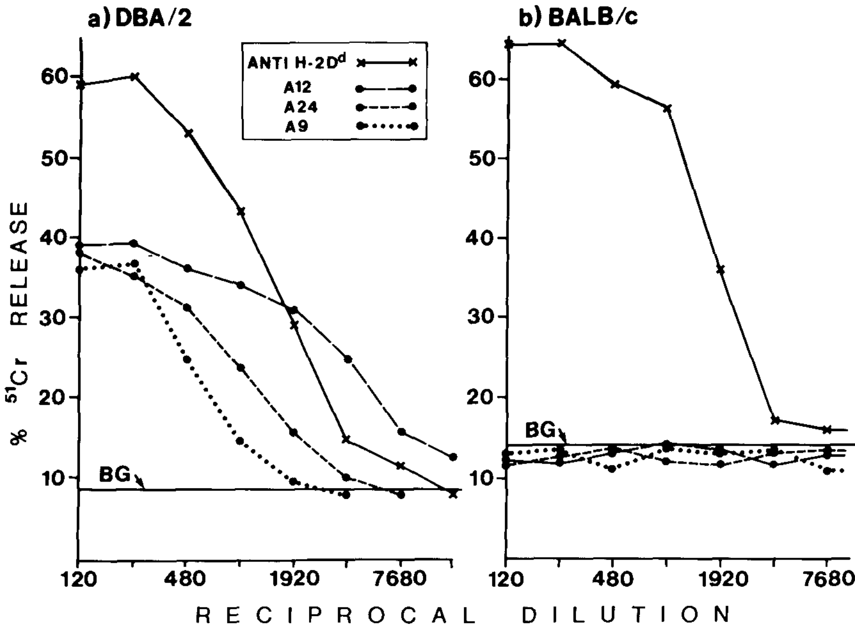


**Fig. 1.** Complement-dependent cytotoxicity ($^{51}$Cr release) of antisera A12, A9, and A24 against LNC of DBA/2 or of BALB/c (specificity control). An anti-H-2D$^d$ serum (A8) is included as a positive control. BG = Background with complement alone

lymphocytes of certain Ly-4.2 negative strains, with a strain distribution and tissue distribution suggesting Ly-6.2. (Because of the anti-Ly-4.2 activity, the Ly-6.2 experiments using A12 have been performed with Ly-4.2 negative strains). In addition, sera with no Ly-4.2 activity have now been produced against the same specificity in two strain combinations (CBA × BALB/c)$F_1$ anti DBA-2 (designated 'A24') and (BALB/c × A)$F_1$ anti DBA/2 /designated 'A9'). A9 contains a second activity against an Ly-6.2 negative strain, CBA/J, and was always rendered Ly-6.2 specific by absorption with CBA/J spleen before use. The titers of these antisera against lymph node cells of an Ly-6.2 positive strain (DBA/2) and an Ly-6.2 negative strain (BALB/c) are shown in Figure 1, compared to an H-2 antiserum. All sera were preabsorbed with recipient spleen cells (and bone marrow cells in some cases) to remove autoantibody.

The antigenic strength of Ly-6.2 is underlined by the high cytotoxic titers of the antisera raised against this antigen (usually greater than 1:1000). Moreover, antibody activity could be detected after only two immunizations, just as observed with an H-2 specificity, so that prolonged and intensive immunization is not required to obtain antibody against Ly-6.2. Both of these features indicate that the antigen Ly-6.2 is a strong antigen in these strain combinations.

### *Genetic Studies*

Segregation analyses of the reactions of A12 were performed to determine the number of genes controlling the cytotoxic reactions of this serum. These tests were performed against peripheral blood lymphocytes, but confirmation with LNC was obtained in some cases. The results showed firstly that the antigen is inherited in a dominant fashion, since the reaction of A12 with (A/J × DBA/2)$F_1$ was positive (data not shown). Secondly, as shown in Table 2, the reaction of serum A12 in the combination C3H × DBA/2 segregated as a single autosomal dominant specificity in the $F_2$ generation. The observed percentage of positive animals (76%) was in good agreement with the expected number of positives for a single segregating gene (75%). In the backcross generations (BC) of the cross (A × DBA/2) × A, the results in the first four backcross generations support the concept of a single segregating gene, rather than two or more segregating genes.

When possible linkage with genes controlling H-2, coat color, or sex was tested, the gene controlling the antigen identified by A12 was found to be unlinked to the genes for these traits.

**Table 2.** Segregation Analysis of Antiserum A12

| Strain Combinations | Generation Tested | Cytotoxicity Results | | | Expected % Positive | $\chi^2$ |
|---|---|---|---|---|---|---|
| | | No. Tested | No. Positive | % Positive | | |
| C3H × DBA/2 | $F_2$ | 37 | 28 | 76 | 75 | 0.01(ns)[a] |
| (A × DBA/2) × A | BC1 | 41 | 17 | 41 | 50 | 1.20(ns) |
| (A × DBA/2) × A | BC2 | 25 | 12 | 48 | 50 | 0.04(ns) |
| (A × DBA/2) × A | BC3 | 33 | 13 | 39 | 50 | 1.48(ns) |
| (A × DBA/2) × A | BC4 | 9 | 5 | 56 | 50 | 0.11(ns) |
| | Total BC | 108 | 47 | 44 | 50(NS) | 1.81(ns) |

[a] ns: not significant

**Table 3.** Correlation Between Various Sera in First Generation Backcross

| Serum | Microcytotoxicity Against Lymph Node Cells | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | A (negative control) | | DBA/2 (positive control) | | A12 negative BC1 | | A12 positive BC1 | |
| | No. positive | % kill[a] | No. positive | % kill | No. positive | % kill | No. positive | % kill |
| Control | — | 5 (2) | — | 7 (1) | — | 5 (1) | — | 9 (1) |
| A12 | 0/4 | 6 (.6) | 4/4 | 42 (6) | 0/6 | 5 (1) | 12/12 | 38 (2) |
| A24 | 0/4 | 6 (2) | 4/4 | 44 (8) | 0/6 | 5 (1) | 12/12 | 37 (3) |
| A9 | 0/4 | 7 (2) | 4/4 | 31 (10) | 0/6 | 7 (1) | 12/12 | 30 (2) |
| A25K | 0/4 | 4 (1) | 4/4 | 38 (6) | 0/6 | 5 (1) | 12/12 | 30 (3) |
| A17K | 0/4 | 6 (1) | 4/4 | 27 (10) | 0/6 | 8 (1) | 12/12 | 29 (2) |

[a] Mean % cytotoxicity, with standard error in parenthesis

### *Segregation of Cytotoxic Activity in Various Sera*

The various cytotoxic reactions of A12, A24, and A9, as well as the sera produced by kidney immunization (see below) could have been produced by different specificities. Accordingly, we tested a number of positive or negative backcross animals with each of these sera. Two bleedings of sera produced by kidney immunization in the same combination as A12 (see below) have been designated A17K and A25K. The reactions of all of the sera were concordant in these animals, as shown in Table 3. Thus each of these sera detects an antigen whose genetic control is linked to that detected by A12, and is probably the same as the antigen detected by A12.

### *Strain Distribution*

Sera A12 and A24 gave positive cytotoxic reactions with strains B10.A (and its congenic partners), C57BL/6, C57BR, AKR, DBA/1, DBA/2, SJL, SWR, RF, and 129. The sera killed 40–70% of LNC with titers of 1 : 1000 in microcytotoxicity in all positive strains, except for the reactions of A12 with Ly-4.2 positive strains, which gave 90% kill. Apart from these strains the reactions of A12 and A24 were similar against all of these strains. Negative strains included A, BALB/c, CBA, C3H, NZB, and HTG. This distribution is identical to that described for Ly-6.2. Furthermore, the possibility that the activity in these sera was directed against specificity Ly-8.2 was eliminated by the strong reactions against DBA/1, which is known to be Ly-8.2 negative (Frelinger and Murphy 1976). Absorption studies revealed that DBA-1 completely removed the activity in A12 or A24 reacting with DBA/2, indicating that all of the antibody in A12 and A24 reacting with DBA/2 is Ly-6.2-like, and that no activity against Ly-8.2 participates in these reactions.

### *Distribution in Lymphoid Tissues*

In microcytotoxicity, A12, A24, and A9 killed 50–80% of LNC, 30–40% of spleen cells, 40–50% of peripheral blood lymphocytes, and 10% of thymocytes. This distribution is similar to that described for Ly-6.2.

**Table 4.** Cytotoxicity of Anti-Ly-6.2 Sera Against T and B Lymphocytes

| Serum tested | % Specific Kill Against Cells from:[a] | | | |
|---|---|---|---|---|
| | BALB/c | DBA/2 | B10.BR | B10.A |
| NMS | <5 | <5 | <5 | <5 |
| A12 | <5 | 65%T | 45%T | 70%T |
| | | 50%B | 22%B | 60%B |
| A24 | <5 | 40%T | 45%T | 50%T |
| | | 12%B | 20%B | 20%B |
| A9 | nt[b] | nt | 38%T | 42%T |
| | | | 17%B | 12%B |
| anti-Ly-4.2 (A22) | nt | nt | 54%T | 65%T |
| | | | 65%B | 68%B |
| anti-Thy-1.2 (B9) | 62%T | 60%T | 60%T | 84%T |
| | 0%B | 0%B | 0%B | 0%B |
| anti-Ia (B1) | 15%T | 10%T | nt | 23%T |
| | 100%B | 100%B | | 100%B |
| anti-H-2 (A8) | nt | nt | nt | 95%T |
| | | | | 94%B |

[a] The % B cells ('green caps') for each cell population was BALB/c 32%; DBA/2 39%; B10.BR 38%; B10.A 33%
[b] nt = not tested

### *Expression on T and B Lymphocytes*

Using an assay originally described for human cells (Van Rood *et al.* 1976) and adapted by us for use with mouse cells, we reexamined the reported specificity of Ly-6.2 for T lymphocytes. Our assay distinguished B lymphocytes from non-B lymphocytes by first treating the target cell population with FITC-labeled anti-immunoglobulin, then incubating these cells so that the B lymphocytes which had bound anti-immunoglobulin formed green fluorescent caps. The cells could then be incubated with antiserum and complement and the dead cells could be detected by their uptake of the red fluorescent dye ethidium bromide. Thus the percentage of dead B cells (the red cells with green caps) and non-B cells (red cells without caps) could be counted.

The results (Table 4) confirm the specificity of the assay for B cells and non-B cells (here designated T cells). The anti Thy-1.2 control killed the great majority of non-B cells, but no B cells. This confirms that the capped cells were indeed all Thy-1.2 negative, and that the anti-immunoglobulin was not being picked up by T-cell Fc receptors. The anti-Ia control killed all the B cells and a small population of non-B cells, as expected (Frelinger *et al.* 1974). The Ly-4.2 serum killed both B and non-B lymphocytes, in agreement with a recent reevaluation of this specificity (Gani and Summerell 1977). When the anti-Ly-6.2 sera A12, A24, and A9 were studied, predominant killing of non-B cells was found, but some B-cell killing (10–20% or more) was invariably observed. Thus on lymph node lymphocytes Ly-6.2 is expressed predominantly on T cells, but is also expressed on at least 10–20% of B (Thy-1.2 negative, surface immunoglobulin positive) lymphocytes.

### *Kidney Expression*

To estimate the relative amount of Ly-6.2 in kidney compared to lymphoid tissue, serial absorption studies of sera A12 or A24 were performed, using equal packed

volumes of either kidney or spleen tissue. Serum aliquots were titrated after each absorption. From this procedure, the slopes of the regression lines for the log reciprocal titers after each absorption were calculated. These slopes provide an estimate of the relative efficiency with which each of these tissues absorbs the anti Ly-6.2, and thus the relative amount of antigen in each of these tissues. As shown in Figure 2, absorption with aliquots of packed A kidney or spleen (negative control) had little effect on the titer of A12 (slopes –0.03 to –0.04). However, sequential absorptions with aliquots of packed DBA/2 kidney or spleen progressively reduced the titer of A12. In repeated experiments, packed kidney tissue was at least as effective as packed spleen cells in absorbing A12 or A24 on a volume for volume basis (slope of –0.83 for kidney compared to –0.43 for spleen). We concluded that the expression of this antigen by kidney cells was at least as extensive as its expression in spleen. Indeed, in the majority of experiments, as in this one, the renal expression of Ly-6.2 appeared to exceed the expression of Ly-6.2 in spleen (per unit volume of packed tissue).

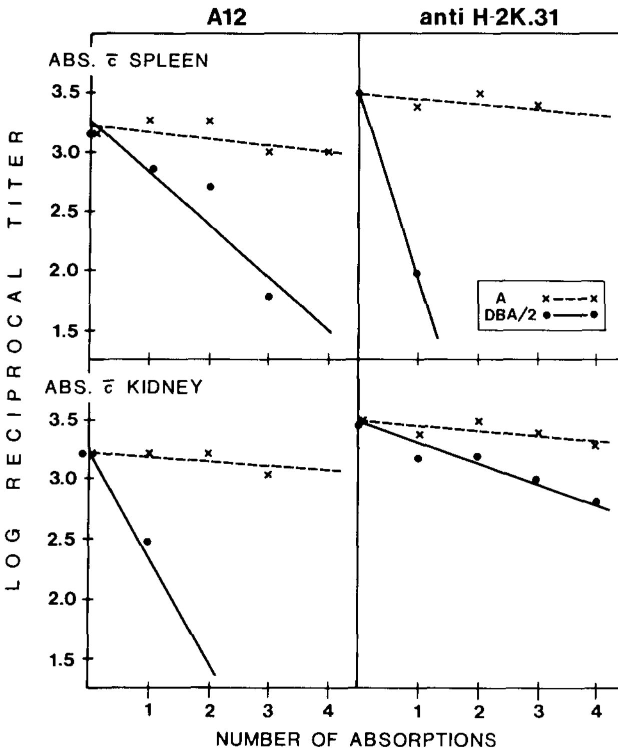


**Fig. 2.** Sequential absorptions to compare the absorptive capacity of kidney to that of spleen for A12 and for an anti-H-2 serum (D-31), showing the effects of four serial absorptions by kidney or spleen from strain DBA/2 or strain A (negative control). Aliquots after each absorption were tested in $^{41}$Cr release against DBA/2 target cells to estimate the titer point (dilution giving 50% of maximum specific release). The log reciprocal titer was then plotted against number of absorptions, and the regression lines were calculated and drawn. The slopes obtained with DBA/2 tissues were: spleen absorbing A12, –0.43; kidney absorbing A12, –0.83: spleen absorbing H-2, –1.52; kidney absorbing H-2, –0.15. Slopes after absorption with A tissues were –0.03 to –0.04

In the same experiments, an additional comparison was made between the number of Ly-6.2 antigen sites in kidney and the number of sites for an H-2 specificity in kidney. Figure 2 shows that the absorption of the H-2 specificity by kidney was relatively inefficient, being only 1/10 as effective as the absorption of the H-2 specificity by spleen (slope –0.15 for kidney compared to –1.52 for spleen). This agrees closely with earlier comparisons of the absorptive capacity of kidney and spleen for anti-H-2 (Pizzaro *et al.* 1961, Amos *et al.* 1963, Graziano and Edidin 1971). Thus, whereas kidney was equivalent to or better than spleen in absorbing the activity of A12, spleen was approximately ten times as efficient as kidney in absorbing anti-H-2 activity. When the efficiency of serial kidney absorptions of A12

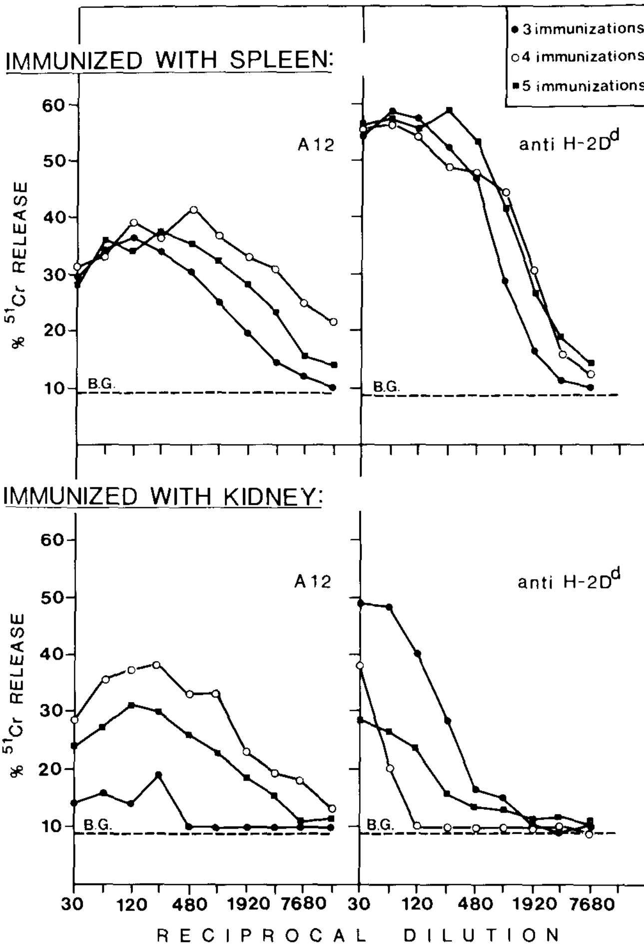


**Fig. 3.** Relative immunogenicity of kidney compared to spleen. Cytotoxic tests ($^{51}$Cr release) of antisera raised by immunizing 3, 4, or 5 times with either spleen or kidney tissue, tested against DBA/2 LNC. A12 sera were raised by injecting B10.A tissues into (BALB/c × A)$F_1$. Two later bleeds of the kidney immunizations in this experiment were designated A17K and A25K (see Table 3). The anti-H-2D$^d$ sera were raised by injecting B10.A(5R) tissues into (B10 × C3H.OH)$F_1$

was compared to the effect of similar absorptions on anti-H-2, the kidney absorbed the A12 activity more efficiently than it absorbed the anti-H-2 (–0.83 for A12 compared to –0.15 for anti-H-2). In other experiments, the kidney consistently absorbed the anti-Ly-6.2 activity as well as or better than it absorbed the anti-H-2 activity using several anti-H-2 sera. As discussed below, this evidence suggests that the amount of antigen Ly-6.2 expressed in kidney is greater than or equal to the amount of an H-2 antigen in kidney.

Ly-6.2 was shown to be expressed in kidney in an immunogenic form. (BALB/c × A)$F_1$ animals were repeatedly injected i.p. with equal volumes of B10.A kidney tissue or spleen tissue. To compare the results with those for an H-2 antigen (B10 × C3H.OH)$F_1$ animals were repeatedly injected with B10.A(5R) spleen or kidney tissue. The results (Fig. 3) show that both anti-Ly-6.2 activity and anti-H-2 activity could be identified after the third, fourth, and fifth immunizations with either spleen or kidney. Although for neither specificity was the kidney immunization as effective as the spleen immunization (possibly because kidney tissue had a very low viability), the relative immunogenicity of Ly-6.2 in kidney compared to spleen was at least as great as the immunogenicity of the H-$2D^d$ specificity in kidney compared to spleen. Thus Ly-6.2 is extensively expressed in and is immunogenic in kidney, and compares favorably in its quantity and immunogenicity with an H-2 specificity.

### *Ly-6.2 Expression in Other Tissues*

In agreement with earlier reports (McKenzie *et al.* 1977), we found that antibody against Ly-6.2 cannot be absorbed by liver or brain. Figure 4 shows that under conditions in which absorptions by kidney or spleen rapidly clear the Ly-6.2 activity, repeated absorptions by brain and liver have no specific effect. This result was consistently demonstrable in other experiments, suggesting that expression of Ly-6.2 in liver and brain is either below the sensitivity of absorption analysis or absent. Similar negative absorptions were obtained with erythrocytes, platelets, skeletal muscle, heart, ovary, or testis.

### *Expression on Nonlymphoid Bone Marrow Cells*

A previous report states that Ly-6.2 is expressed in bone marrow, presumably due to T lymphocytes in that tissue (McKenzie et al. 1977). The Ly-6.2-like specificity detected by our sera A9, A12, and A24 is similarly expressed in bone marrow, but considerably in excess of that previously reported, and far in excess of what could be attributed to T lymphocytes in bone marrow. A comparison of the cytotoxic reactions of various sera against BMC is shown in Figure 5. As can be seen, our sera against Ly-6.2 consistently produced much more $^{51}Cr$ release against BMC than did anti-Thy-1.2. Indeed, in the experiment shown the anti-Thy-1.2 serum was negative. In similar microcytoxicity studies 40–50% of BMC were specifically killed by Ly-6.2, and many of these cells were target cells of obvious myeloid rather than lymphoid appearance. Interestingly, in many experiments, Ly-6.2 sera killed as many BMC as did an H-2 specificity, and invariably far more than the Ia or Thy-1.2 controls. We conclude that the expression of Ly-6.2 in BMC cannot be due to T lymphocytes in this tissue.

Further experiments show that BMC can completely absorb the activity in A12

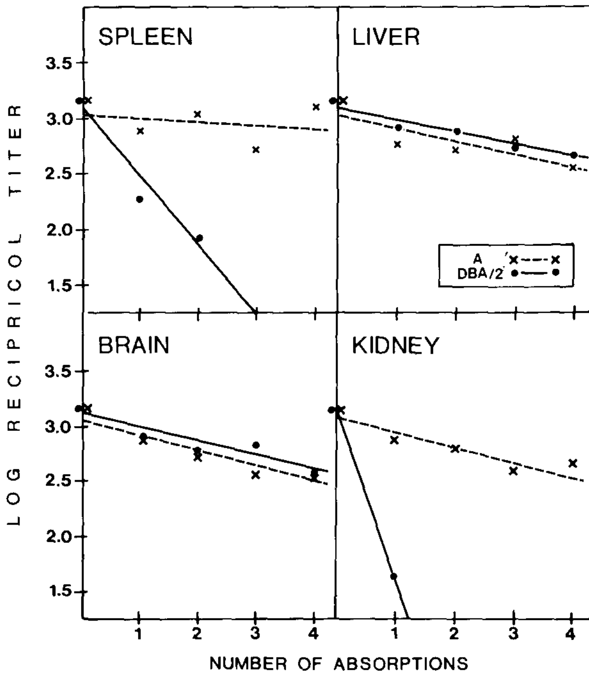


**Fig. 4.** Sequential absorptions to compare the relative ability of DBA/2 spleen, liver, brain, and kidney suspensions to absorb the activity in antiserum A12. Strain-A tissues were included as negative controls. Method and expression of data as in Figure 2

against LNC and spleen cells, and LNC can absorb the activity directed against BMC. This is evidence that the reaction of A12, A24, and A9 against bone marrow cells is caused by same antigen that causes activity against peripheral lymphocytes.

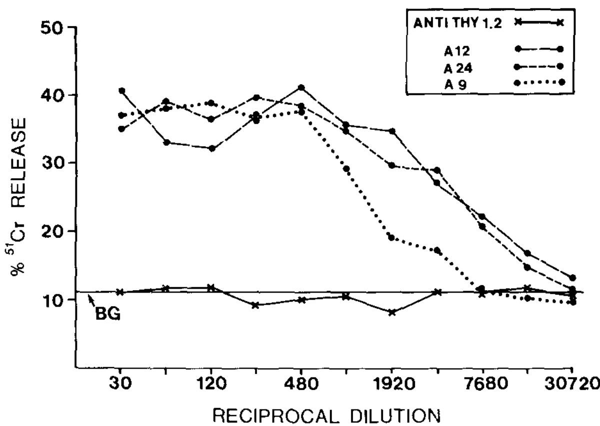


**Fig. 5.** Cytotoxic tests with A9, A12, and A24 against DBA/2 bone marrow cells. Anti-Thy-1.2 (B9) was included as a control

**Table 5.** Segregation of Bone Marrow Cytotoxic Activity

| Serum Tested | Positive Backcross Animals | | | Negative Backcross Animals | | |
|---|---|---|---|---|---|---|
| | No. tested | % kill[a] | | No. tested | % kill | |
| | | bone marrow | lymph node | | bone marrow | lymph node |
| Control[b] | 10 | 9(1) | 13(2) | 4 | 9(1) | 11(4) |
| A12 | 9 | 54(6) | 41(4) | 3 | 7(2) | 14(2) |
| A24 | 9 | 58(3) | 40(3) | 2 | 8(2) | 14(2) |

[a] Mean % cytotoxicity, with standard error in parenthesis; microcytotoxicity assay
[b] Complement alone

Additional evidence that the antigen on BMC is identical to the antigen on LNC has been obtained by testing the positive and negative BCl animals: the strong bone marrow reaction segregated identically with the lymphoid reaction in 10 positive and 4 negative animals (Table 5). Thus 40–50% of bone marrow cells were lysed by our anti-Ly-6.2 sera, and the bone marrow antigen segregates with and is probably identical to that on peripheral lymphocytes.

## *Expression of Ly-6.2 on a Nonlymphoid Tumor*

A previous report found that Ly-6.2 is expressed on a T-cell tumor but not on a B-cell tumor (McKenzie *et al.* 1977). In our studies we tested the DBA/2 mastocytoma tumor P815Y, which is a nonlymphoid tumor (Dunn and Potter 1957). The P815Y was killed by antisera A12, A24, and A9 (titers > 1 : 1000), but not by anti-Thy-1.2. In addition, the P815Y absorbed the antibody activity in these sera (Fig. 6) proving that the activity killing the P815Y cells is likely the same as that killing the

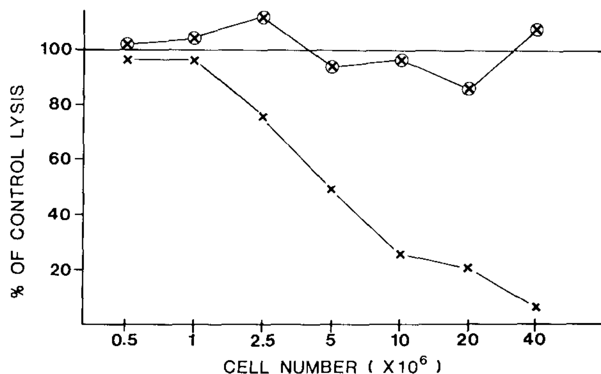


**Fig. 6.** Absorption of antiserum A12(×) by increasing numbers of P815Y cells. Absorption of anti-Thy-1.2(⊗) was included as a control, since P815Y is Thy-1.2 negative. Cells were incubated at 20° C for 60 minutes with 50 $\mu$l of diluted sera (1:50 for A12, 1:10 for anti Thy-1.2, the dilutions being determined by the ratio of their cytotoxic titers). Absorbed sera were tested against DBA/2 LNC in the $^{51}$Cr release assay

lymph node lymphocytes, and was not merely due to antiviral or antitumor activity in these sera. This absorption was specific, in that anti-Thy-1.2 was not absorbed by the tumor cells. This control was necessary to prove that the P815Y cells were not absorbing by their Fc receptors, and that contaminating T lymphocytes were not responsible for the absorption.

## Discussion

Some properties of Ly-6.2 as a lymphocyte alloantigen have been described previously (McKenzie *et al.* 1977), and the present results confirm and extend these findings. Our antisera raised in Ly-6.2 disparate strain combinations react against a single antigen controlled by genes unlinked to *H-2*. This antigen has an identical strain distribution and a similar tissue distribution to those of Ly-6.2, and the antisera A12 and A9 are raised in the same $F_1$ as the original serum. Thus there is little doubt that the antigen we have detected with A12, A24, and A9 is Ly-6.2. In addition, Ly-6.2 may be identical to the specificity designated 'DAG' (Sachs *et al.* 1973) which is also known to have nonlymphoid expression (J. Sachs, personal communication). If identity could be established, this would give priority to the name DAG for this antigen. However, we have studied an antiserum similar to that used by these authors (BALB/c anti DBA/2) and have been unable to find cytotoxic activity. Hence the relationship of DAG to Ly-6.2 remains in doubt.

The distinctive features of the specificity described in the present report are its antigenic strength and tissue distribution. The immune response against this antigen is unusually strong for a non-H-2 antigen, both in the early appearance of antibody after only two immunizations, and in its cytotoxic titer (1 : 1000) against peripheral lymphocytes. More importantly, the nonlymphoid expression of this antigen is unique, and argues against continuing to classify it as an Ly antigen, as we discuss in the 'Introduction.' Optimally, we would like to see this specificity given a more appropriate name recognizing its strong nonlymphoid expression. However, in accord with the conventions for the non-H-2 antigens (Boyse *et al.* 1977) and Snell's requirements for a good genetic symbol (Snell 1977), it is probably advisable to retain the Ly-6.2 designation for the sake of a simple genetic nomenclature.

The demonstration of the B-cell expression of Ly-6.2 contradicts the previous suggestion that this antigen might be specific for T lymphocytes. This recalls a similar sequence of events for Ly-4.2, which was originally thought to be B-cell specific (McKenzie and Snell 1975), but has since been shown to be expressed on both T and B cells (Gani and Summerell 1977). The technique described by Van Rood and coworkers for detecting specificities on T and B cells is an important advance in this respect, and we suggest that this method is the best available one for readily determining B- and non-B-cell expression of a mouse alloantigen. Our own experience, like that of Gani and Summerell (1977), suggests that the details of the complement lysis assay and complement batch may have a significant impact upon the demonstration of an alloantigen in various lymphocyte subpopulations. This must be considered before assigning LyT and LyB designations, and strongly implies that a variety of complement sources, cytotoxicity assays, and investigations in different laboratories are required before exclusive T- or B-cell expression can be regarded as proven.

When studying alloantigen expression and immunogenicity in kidney, one must consider whether the alloantigen is carried on leukocytes or on parenchymal cells. In

the case of Ly-6.2, the present study shows that this antigen is expressed at least as extensively in renal tissue as in lymphoid tissue, which argues that the renal tissue expression is not merely due to leukocytes in that tissue. Moreover, we have demonstrated in unpublished studies that anti-Thy-1.2 is not specifically absorbed by similar preparations of renal tissue, as would be expected if T lymphocytes had a role in the specific absorption of anti-Ly-6.2 by kidney. Other experiments have shown that residual blood in the kidney cannot account for the absorption of A12 by kidney: even whole blood absorbs poorly compared to equal volumes of packed kidney. Thus the absorption of anti-Ly-6.2 by kidney cannot be attributed to T lymphocytes resident in the kidney or to leukocytes present in blood in the kidney. However, the effectiveness of renal tissue in provoking antibody responses to this antigen may be due to passenger leukocytes. Although we cannot refute this, we point out that the immunogenicity of Ly-6.2 in kidney compares favorably with that of an H-2 antigen, and is as likely to be mediated by passenger leukocytes as is the immunogenicity of H-2. We believe that the antibody response against this antigen in renal tissue is due to antigen expression on kidney parenchymal cells, but the debate over passenger leukocytes is beyond the scope of this discussion.

In our experiments we employed sequential absorption to compare the relative amount of an H-2 antigen and Ly-6.2 in kidney (Fig. 2). This comparison is based on the unverifiable assumption that the relationship between the cytotoxic titer and the number of antibody molecules binding for two specificities is relatively constant. Thus even though the absorbing tissue, target cells, and cytotoxicity test conditions for the two sera were identical, and even though we repeated this experiment with A12, A24, and several anti-H-2 sera, the results of such experiments are suggestive rather than conclusive. Bearing in mind this caveat, our results still imply clearly that the number of Ly-6.2 antigen sites in kidney is greater than or equal to the number of sites for an H-2 specificity. This finding highlights the importance of documenting the immunogenicity and histocompatibility effect of this antigen, both in its own right and because it might provide insight into the strength and histocompatibility effect of minor histocompatibility antigens in kidney.

Unfortunately, the mouse tends to retain kidney allografts, even when these are *H-2* incompatible (Russell *et al.* 1978) and is therefore not a very suitable model for studying the histocompatibility effects of Ly-6.2 in kidney. Nevertheless, a human antigen comparable to Ly-6.2 in its strong induction of humoral immunity and its extensive renal expression may be of considerable clinical significance. In clinical renal transplantation, humoral immune mechanisms can cause or contribute to graft loss (Carpenter *et al.* 1976), and some non-HLA antigens (e.g., ABO and MN) are known to influence allograft survival. Experimental confirmation of the importance of non-major histocompatibility loci in kidney transplantation has been obtained in the rat (Thoenes *et al.* 1974, Thoenes 1975, Paris *et al.* 1978). Thus it is reasonable to believe that a human analog of Ly-6.2 in a transplanted kidney could serve as a target for a strong destructive (or protective?) humoral response. Antigen Ly-6.2 should be a useful model in the search for analogous human specificities with renal and lymphoid expression. Similar considerations also apply to the potential importance of a human analog of Ly-6.2 in bone marrow transplantation. Of interest in this respect is the observation that human bone marrow rejection due to serologically identifiable alloantigens unlinked to HLA is a frequent occurrence.

Finally, the nonlymphoid expression of non-H-2 alloantigens may be an avenue by which immunogenetics will have an impact on disciplines other than immunology. It is difficult to underestimate the contribution that markers such as

Thy-1 and the Ly antigens have made to the understanding of the development, structure, function, and diseases of lymphoid tissues (Raff *et al.* 1971, Cantor and Boyse 1977). The possibility that markers such as Ly-6.2 with selective expression in kidney and bone marrow may assist in the study of these tissues deserves further consideration. For example, Ly-6.2 should be investigated for its cellular localization in kidney, its relationship to kidney transport or other functions, and for its possible influence upon spontaneous or induced immunologic renal disease.

*Acknowledgements.* We wish to thank the National Institutes of Health for providing certain antisera, Dr. C. S. David for providing certain breeding animals, Mrs. Marilyn Kapeluto for her excellent typing, and Mr. Arnold Risdell for assistance with the illustrations. This work was supported by grants from the Kidney Foundation of Canada, the Medical Research Council of Canada, and the Mount Sinai Hospital.

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*Received August 8, 1978; revised version received September 14, 1978*