Ly-4, A New Locus Determining a Lymphocyte Cell-Surface Alloantigen in Mice

(antibody/node and thymus lymphocytes)

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ABSTRACT A new locus is described that determines an alloantigen on the surface of lymphocytes. It differs from loci previously described in that the corresponding antibody, at least in most antisera, reacts almost exclusively with node lymphocytes, and weakly or not at all with thymus lymphocytes. Positive strains include C57BL/6, C57BL/10, C57L, C57BR/cd, and RF; negative strains include BALB/c, C3H, and SJL. The symbol Ly-4 is assigned, with the C57BL/6 allele being Ly-4, and the BALB/c allele Ly-4.

A dozen loci are known in mice that determine alloantigens on the surface of red cells or lymphocytes (for reviews, see refs. 1 and 2). Seven red cell alloantigen loci have been described, designated Ea-1 to Ea-7 (3-10). The Tla locus (11) determines an alloantigen confined to thymus lymphocytes. The antigen of another locus, Thy-1 or θ (12), is a useful marker for thymus-derived or T lymphocytes; unlike the antigen determined by Tla, it persists after the lymphocytes leave the thymus. The antigens of Ly-1 (13, 14) and Ly-2 (15) (13) occur on node and spleen, as well as thymus, lymphocytes but, like the Tla and Thy-1 antigens, are most easily studied with lymphocytes from the thymus. An alloantigen of plasma cells is determined by the Pca locus (16). The major histocompatibility locus (or complex) of the mouse, H-2, not only is important in graft rejection, but is also both a blood group and a lymphocyte group locus (16), therein holding a unique position among histocompatibility loci.

The numerous inbred and congeneric (17) mouse strains, typed for the known alleles of the various cell-surface alloantigen-determining loci (2), make it possible to set up recipient–donor combinations for the production of antisera that should yield specific, known antibodies, or antibodies that can be identified at once as unknown. Tested against lymphocytes by the dye exclusion (18) or chromium label (19) cytotoxic methods, the unknown antibodies might be expected to expand an interesting new group of loci. Because tests with antisera in which non-H-2 antibodies were not excluded suggested that unknown non-H-2 antibodies may be common, we decided to institute a planned search for such antibodies. We have found that antibodies are produced by the majority of recipient–donor combinations selected to exclude known reagents. Most of the antibodies are weak, some too weak to work with. Analysis is sometimes complicated by the number of antibodies present, and by a tendency to low maximum lysis (suggestive of reaction with a restricted category of lymphocytes), but several workable antisera have been obtained. We report here antisera which identify a new lymphocyte alloantigen locus. The locus is distinct from non-H-2 loci heretofore described in that its antigen is more readily demonstrated on node than on thymus lymphocytes.

METHODS

Mice. Mice came from the personal colonies of Snell or Bailey or from the Production Department of The Jackson Laboratory. For some strains, frequently referred to, we shall use the standard abbreviations. Thus: C57BL/6 = B6, C57BL/10 = B10, BALB/c = C, DBA/2 = D2.

Antisera. Antisera were usually produced in hybrid recipients, since this procedure assists in the exclusion of unwanted antibodies. Donor cells for injection were prepared in the cytovin (20) in Hank’s solution. Mice were injected intraperitoneally with donor thymus, 1 donor (preferably 3 to 5 weeks-old) per 25 recipients, at 8+ weeks of age, and again with the same type of cell suspension 40 days later. Subsequent injections were a mixture of thymus, spleen, and submaxillary gland from different, 1 donor per 10 recipients. The first bleeding was usually made 7–10 days after two such injections given at weekly intervals. Mice were bled from the tail. After a titer was established, mice were injected at 14-day intervals and bled on days 7 and 10 of the intervening weeks. Up to 16 bleedings were obtained per mouse, but the cytotoxic titers were often highest in the earlier bleedings. Thymus cells were used for the first two injections because of evidence that of all the lymphoid tissues they are least likely to induce tolerance (21, 22), and because thymus cells are effective inducers of graft immunity for most or all H loci (23). The interval of 40 days between first and second injections was selected because the capacity for a second set response continues to rise for about this period of time (24-26).

Submaxillary salivary gland, as well as spleen, was added in later injections because it contains H-2 antigens (27) and is quickly dissected. When reports appeared that a substance or substances in the submaxillary gland may destroy or alter lymphocytes and affect the immune process (28-31), we ran some tests of the influence of its inclusion or exclusion on the antibody response, and satisfied ourselves that it at least does not reduce—and may sometimes improve—antibody production.

The antisera used are shown in Table 1. The antibodies in the antisera possessing known non-H-2 antibodies have been identified by an analysis of their strain distribution patterns and, in the case of the lymphocytotoxic antisera, by their relative specificities with node, as compared with thymus, lymphocytes. The antibody in the anti-Ea-θ antisera was
also tested by running a linkage test of reactivity with the agouti locus (32).

**Cytotoxic Test.** The chromium-label cytotoxic test, as we use it, has been fully described (16). Target cells were node or thymus lymphocytes. Rabbit serum was the source of complement. The titer was calculated as the antiserum dilution that yielded 80% of the maximum lysis.

**Hemagglutination Test.** For hemagglutination tests, we used a modification of the poly(vinylpyrrolidone) method of Stimpfling (16).

**Genetic Methods.** Two methods were used to test the identity or nonidentity with known loci of the locus under study.

The first was the typing of backcrosses. (We use backcross in the genetic sense, to mean the crossing of F1 hybrids to the multiple recessive.) Two backcrosses were used: (B6 x RF) x HTG and (B6 x C3H) x C3H. The second of these was continued into later backcross generations to introduce genes from B6 onto a C3H background. Relevant data were obtained from generations N2 through N5.

The second method made use of recombinant-inbred (RI) strains (33). These are inbred strains produced by brother-sister inbreeding after a cross between two established inbred strains. The alleles at every locus by which the parent strains differ become fixed in patterns which are characteristic of the individual loci. If a locus under test has a different pattern from a known locus, it of necessity is different. Identical patterns establish a presumption of identity, but may rarely be fortuitous. The more loci that are tested, the greater the chance of finding a significant match with any new locus. The RI lines thus function as a "frozen" recombinant generation, to which one can continually have recourse for new tests. The seven RI lines used came from an initial C x B6 cross (33), and are designated CXBD, CXBE, CXBG, CXBH, CXBI, CXBJ, and CXBK.

**RESULTS**

Several antisera produced in recipient-donor combinations that could not yield antibodies against known lymphocyte surface alloantigens nevertheless contained lymphocytotoxic antibodies. Some of these were obviously complex, but served to suggest modifications of the recipient-donor combination that should, and sometimes did, yield more interpretable antisera. One antibody has now been thoroughly characterized. It defines a new locus, which we call Ly-4.

Five of the antisera in which it occurred are shown in Table 1. Of these, AS-586, (BALB/c x SWR) anti-B10.D2, appeared to be the most nearly monospecific, and we have adopted it as the standard. A repeat antiserum showed essentially identical properties, though with the suggestion of a weak contaminant not present in the original. An anti-Ea-4.2 was present in addition to the lymphocytotoxic antibody, but this additional antibody is reactive only with erythrocytes.

Of the cytotoxic activity of AS-586 was almost exclusively with node lymphocytes; reactions with thymus lymphocytes were negative or very weak, especially in the early bleedings. AS-605, (BALB/c x A.BY) anti-CXBJ, and AS-661, (DBA/2 x SWR) anti-B10.D2, were very similar, but AS-605 was generally weak and AS-661 contained a weak, but unidentified, contaminant. AS-564, (DA x 129) anti-B10, reacted with thymus as well as node lymphocytes of the donor, and of at least some other positive strains. It also gave a low-titered reaction with strain CXBH not shown by AS-586, and hence is probably indicative of a contaminant antibody. AS-569, (A x CE) anti-C57BR/cd, contained a second, strong node cytotoxic antibody which we have partly characterized.

The pattern of strain reactivity, as inferred primarily from results with AS-586 and its repeat, but also in part from results with the other antisera, is as follows:


Titers with positive node lymphocytes were usually of the order of 1/40, with a good maximum lysis. Positive strains C57BR/cdJ, C58/J, MA/J, and RF/J consistently gave a considerably lower maximum lysis, and often a lower titer. These are all H-2k strains, suggesting that there may be an interaction between H-2k and Ly-4. This suggestion has been checked in two ways. The (B6 x C3H) x C3H backcross

<table>
<thead>
<tr>
<th>Antiserum no.</th>
<th>Recipient</th>
<th>Donor</th>
<th>Specificity tested</th>
<th>Source of test cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS-564</td>
<td>DA x 129</td>
<td>B10</td>
<td>Ly-4</td>
<td>Nodes</td>
</tr>
<tr>
<td>AS-569</td>
<td>A x CE</td>
<td>C57BR/cd</td>
<td>Ly-4, Ly-7</td>
<td>Nodes</td>
</tr>
<tr>
<td>AS-586</td>
<td>BALB/c x SWR</td>
<td>B10.D2</td>
<td>Ly-4</td>
<td>Nodes</td>
</tr>
<tr>
<td>AS-605</td>
<td>BALB/c x A.BY</td>
<td>CXBJ</td>
<td>Ly-4</td>
<td>Nodes</td>
</tr>
<tr>
<td>AS-661</td>
<td>DBA/2 x SWR</td>
<td>B10.D2</td>
<td>Ly-4</td>
<td>Nodes</td>
</tr>
<tr>
<td>C-33</td>
<td>B10.D2 x A</td>
<td>B10.A(5R)</td>
<td>H-2.33</td>
<td>Nodes</td>
</tr>
<tr>
<td>AS-546</td>
<td>B10 x LP.RIII</td>
<td>B10.RIII(72NS)</td>
<td>Ea-2.1</td>
<td>Red cells</td>
</tr>
<tr>
<td>AS-531</td>
<td>DBA/2 x RF</td>
<td>B10.D2</td>
<td>Ea-4.2</td>
<td>Red cells</td>
</tr>
<tr>
<td>AS-591</td>
<td>DBA/1 x LP</td>
<td>DA</td>
<td>Ea-6.1</td>
<td>Red cells</td>
</tr>
<tr>
<td>AS-522</td>
<td>129</td>
<td>A.BY</td>
<td>Ea-7.2</td>
<td>Red cells</td>
</tr>
<tr>
<td>AS-526</td>
<td>C3H x DBA/2</td>
<td>CE</td>
<td>Ly-1.2</td>
<td>Thymus</td>
</tr>
<tr>
<td>AS-536</td>
<td>C3H x BDP</td>
<td>B10.Y</td>
<td>Ly-2.2</td>
<td>Thymus</td>
</tr>
<tr>
<td>AS-608</td>
<td>B10.RIII(72NS)xC58</td>
<td>RF</td>
<td>Thy-1.1</td>
<td>Thyus</td>
</tr>
</tbody>
</table>
Tables 2, 3, and 4. Segregation of Ly-4 tested against five known loci in a (B6 x C3H) x C3H backcross, and in subsequent backcross generations made to produce C3H.B6 congenic lines

**Table 2.** Segregation of Ly-4 tested against five known loci in a (B6 x C3H) x C3H backcross, and in subsequent backcross generations made to produce C3H.B6 congenic lines

<table>
<thead>
<tr>
<th>Loci tested</th>
<th>Number of mice of indicated serotype</th>
<th>Percent recombination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ly-4 versus H-2</td>
<td>++* 5 2 4 4</td>
<td>40</td>
</tr>
<tr>
<td>Ly-4 versus Ea-4</td>
<td>+ 1 6 7 5</td>
<td>68</td>
</tr>
<tr>
<td>Ly-4 versus Ea-7</td>
<td>- 7 6 8 8</td>
<td>48</td>
</tr>
<tr>
<td>Ly-4 versus Ly-1</td>
<td>= 1 3 4 2</td>
<td>70</td>
</tr>
<tr>
<td>Ly-4 versus Ly-2</td>
<td>- 7 2 5 6</td>
<td>35</td>
</tr>
</tbody>
</table>

* The first plus or minus refers to results with anti-Ly-4, the second plus or minus to results with antisera identifying the five known loci. Positive reactions entered the cross in coupling.

Segregated for H-2 or H-2a as well as for Ly-4. As shown in Table 2, H-2 clearly segregated independently of Ly-4. Among 11 mice positive for Ly-4 and tested for H-2, strong reactions were invariably associated with the H-2a/H-2a genotype and weak reactions with the H-2h/H-2h genotype. Secondly, in a simultaneous test with AS-564, H-2a strains C57BL/10 and C57L gave, respectively, titers (maximum titer) of 1/24 (77%) and 1/51 (76%), while H-2h strains B10.A and C3HBR gave 1/13 (67%) and 1/15 (61%).

The strains distribution pattern of positive reactions with AS-566 and related antisera and the tendency of these antisera to react exclusively or predominantly with node lymphocytes suggests that they identify a new locus. More positive evidence comes from the backcross tests. The results are given in Tables 2 and 3. Positive reactions with AS-566 segregated independently from H-2, Ea-2, Ea-4, Ea-7, Ly-1, Ly-2, and Thy-1. Since Ly-3 (34) shows complete linkage with Ly-2 (it may be an allele), and Tla shows very close linkage with H-2, tests with these were regarded as unnecessary. Tests were not run against all Ea (erythrocyte antigen) loci, but the marked serological differences make identity unlikely. The same conclusion applies to Pta, the plasma-cell-antigen locus (15).

Additional evidence on independence comes from results with the CXB recombinant inbred lines (Table 4). Unexpectedly, since the backcross data conclusively show independence of the AS-566 locus from Ea-4 and H-2, all these loci have the same CXB pattern. Presumably this identity is a coincidence, though it may possibly indicate some selective advantage of particular allelic combinations. Ea-6 shows a separate pattern, and is therefore distinct. Independence from various histo-

**Table 3.** Segregation of Ly-4 tested against three known loci in a (C57BL/6 x RF) x HTG backcross

<table>
<thead>
<tr>
<th>Loci tested</th>
<th>Number of mice of indicated serotype</th>
<th>Percent recombination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ly-4 versus Ea-4†</td>
<td>++* 4 5 7 4</td>
<td>40</td>
</tr>
<tr>
<td>Ly-4 versus Ea-44</td>
<td>+ 3 6 4 7</td>
<td>50</td>
</tr>
<tr>
<td>Ly-4 versus Thy-1†</td>
<td>= 4 3 4 4</td>
<td>55</td>
</tr>
</tbody>
</table>

* The first plus or minus refers to results with anti-Ly-4, the second to results with antisera identifying the three known loci.
† Positive reactions entered the cross in repulsion.
‡ Positive reactions entered the cross in coupling.

Table 4. Strain distribution patterns of the BALB/c (C) and C57BL/6 (B) alleles of H-2, Ea-4, Ea-6, and Ly-4 loci in the CXB recombinant inbred strains

<table>
<thead>
<tr>
<th>Locus</th>
<th>CXBD</th>
<th>CXBE</th>
<th>CXBG</th>
<th>CXBH</th>
<th>CXBI</th>
<th>CXBJ</th>
<th>CXBK</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-2</td>
<td>C</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ea-4</td>
<td>C</td>
<td>B</td>
<td>B</td>
<td>C</td>
<td>B</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>Ea-6</td>
<td>C</td>
<td>B</td>
<td>C</td>
<td>B</td>
<td>B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ly-4</td>
<td>C</td>
<td>B</td>
<td>C</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td></td>
</tr>
</tbody>
</table>

compatibility (H) loci is also proved. Table 4 does not include published data on H locus patterns (33) nor some recent unpublished data (Bailey), but in every case the pattern is distinct. Identity with H-1, H-7, H-8, and several more recently described H loci is ruled out. Distinctiveness from H-13, which is closely linked with the agouti (a) locus, is indicated by the very distinct CXB pattern given by AS-586 and a. The same possibly applies to H-8, also linked to a, but with more crossing-over. There is no specific evidence concerning other H loci, but the difficulty of producing detectable antibodies against H loci (1), as contrasted with the ease of producing anti-Ly-4, argues for distinctness. An Ly-4 congenic line now under production should permit a final answer to this question.

**DISCUSSION**

The evidence presented here establishes a new lymphocyte alloantigen locus, Ly-4. The antibody which identifies it is not high-titered, but has appeared in several different antisera and appears to be easy to produce. Including the CXB recombinant inbred strains, 35 strains have been typed as reactors or nonreactors. Because, for several non-H-2 alloantigens, the specificity of the widely used C57BL strain and its various sublines has been assigned the number 2, we suggest calling the Ly-4 specificity of C57BL Ly-42, and the corresponding allele Ly-4a. Since C57BL is a reacting strain, and we have not as yet identified the reciprocal antibody, this choice assigns the 1 specificity and the a allele to the non-reactors.

A distinctive feature of some of the anti-Ly-4 sera is that they react in the cytotoxic test exclusively—or almost exclusively—with node, and not with thymus, lymphocytes. There is some evidence that the antisera which show thymic reactions contain additional antibodies, but we cannot exclude the possibility that some variants of anti-Ly-4 are thymus reactive. In any case the antigen is probably on the thymus, since we produce our antisera with priming injections of thymus cells only. One of the known differences between node and thymus lymphocytes which might account for differences in reactivity with different antibodies is that the former include a considerable proportion of bone-marrow-derived or B lymphocytes. One antigenic difference between B and T (thymus-derived) lymphocytes is already well established—the T lymphocytes possess and the B lymphocytes lack the Thy-1 or 3 antigen (36).

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