Qat-4 and Qat-5, New Murine T-Cell Antigens Governed by the Tla Region and Identified by Monoclonal Antibodies*

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Adjacent to the major histocompatibility complex (MHC) genes of the mouse lies a series of genes which code for T-lymphocyte surface antigens. The Tla genes were the first to be described a decade ago (1). The antigen(s) governed by this locus is expressed exclusively on thymocytes and leukemia cells and thus represented the first example for an alloantigen characteristic of a differentiated cell type. Many other differentiation antigens have since been discovered, but no other linkage group than the IXth has shown an equal concentration of genes specifying cell surface antigens. Some of these antigens such as H-2K and H-2D are expressed on most tissues, whereas others display a more restricted tissue distribution. Thus, Ia determinants specified by the I-A and I-E subregion are predominantly expressed on B lymphocytes and macrophages (2), whereas the region between H-2D and Tla codes for a set of serologically defined antigens, termed Qa antigens, of which the Qa-2 and Qa-3 antigens are predominantly found on T lymphocytes of different differentiation stages (3–5). In addition, a set of surface structures encoded by the Tla region was recently identified by the use of cytotoxic T cells (6, 7, and K. F. Lindahl, personal communication).

We report here two new surface antigens of T lymphocytes, Qat-4 and Qat-5, which are governed by genes linked to the Tla/Qa region. The identification of these differentiation antigens was made possible by the discovery of two monoclonal antibodies which arose in hybridomas generated by cell fusion between BALB/c myeloma cells and AKR spleen cells, immune to C57BL/6 lymphocytes. This report is an example of the value of monoclonal antibodies for the detection of new antigenic systems, a feature which has been stressed repeatedly since introduction of the fusion technique by Köhler and Milstein (6).

Materials and Methods

Mice. Mice were purchased from G1. Bomholtgaard, Ltd., Ry, Denmark; OLAC 1976 Ltd., England; The Jackson Laboratory, Bar Harbor, Maine; or were maintained in our own facilities.

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Cell Hybridization. The cell hybridization technique for the production of hybridomas secreting alloantibodies has been described in detail elsewhere (8, 9). In the present case, four female AKR mice were immunized intravenously with a mixture of $2 \times 10^7$ spleen and thymus cells of male C57BL/6 mice. After 3 wk the mice received an additional injection of $3 \times 10^7$ spleen cells and $3 \times 10^7$ thymocytes. 3 d later the spleen cells were hybridized with the myeloma P3-NS-1-Ag4 (a gift of Dr. C. Milstein, Cambridge University) with polyethylene glycol, mol wt 4,000. The fusion mixture was distributed at a density of $5 \times 10^5$ cells per well into 624 cups of Costar 3524 multiwell plates (Costar, Data Packaging, Cambridge, Mass.). Growth of hybrid cells was observed in 308 wells.

Culture supernates were tested for alloantibody activity in a microcytotoxicity assay (9) with C57BL/6 spleen cells as target cells.

Cloning of hybridoma cells from wells displaying cytotoxic activity was performed by the limiting dilution technique (10). For further analysis, either culture supernates of cloned cells were used, or high titered ascites fluids were obtained by intraperitoneal injection of hybridoma cells into either (AKR × DBA/2)F1 or NMRI nu/nu mice which 2 d previously had received 0.5 ml mineral oil intraperitoneally (11).

Serological Methods. Serological analysis of monoclonal antibodies was performed utilizing the microcytotoxicity assay (9) as well as the chromium release assay (3). For indirect immunofluorescence, cells were first incubated with monoclonal antibodies for 30 min at 4°C and subsequently, after washing, with fluorescein-conjugated rabbit anti-mouse Ig (Behringwerke, Marburg/Lahn, Federal Republic of Germany) for another 30 min at 4°C. Fluorescence was evaluated with a fluorescence microscope (Leitz, Wetzlar, Federal Republic of Germany) and with a fluorescence-activated cell sorter (FACS I, Becton, Dickinson FACS Systems, Mountain View, Calif.). Removal of B lymphocytes was performed by either nylon wool fractionation (12) or by use of anti-mouse-Ig-coated plastic dishes (13).

Results

The spleen cells of two AKR mice immune to C57BL/6 lymphocytes were hybridized with the myeloma P3-NS-1-Ag4. Of 624 wells, each seeded with $5 \times 10^5$ cells, 308 showed growth, and two wells, numbers B16-146 and B16-167, contained cytotoxic antibody for C57BL/6 spleen cells. Hybridoma cells from these wells were cloned by the limiting dilution technique. Because all subclones isolated were antibody positive, the original wells contained most likely single, stable clones. Both hybridomas were adapted to growth in ascitic form in either (AKR × DBA/2)F1 or NMRI nu/nu mice which had received 0.5 ml mineral oil 2 d previously. Precipitation of ascitic fluid by ammonium sulfate yielded 14 mg/ml of immunoglobulin for B16-146 and 6.5 mg/ml for B16-167. Fig. 1 shows the cytotoxicity titers of ascites and culture supernates. Accordingly, ascites fluids (Fig. 1A) contain 100- to 1,000-fold higher antibody concentrations than culture supernates (Fig. 1B). Culture supernates of both hybridomas contain immunoglobulins consisting of $\mu$-heavy and kappa light chains, as determined by reaction with class specific anti-immunoglobulin conjugated with alkaline phosphatase, according to a technique developed by J. F. Kearney (personal communication).

Mapping Studies and Strain Distribution. Because, as will be shown below, the two monoclonal antibodies react with T-cell antigens governed by the Qa/Tla region, these antigens have been designated Qat-4 and Qat-5. In Table I, the reactivity patterns obtained with some inbred strains are listed. It can be seen that monoclonal antibodies B16-146 (anti-Qat-4) and B16-167 (anti-Qat-5) react with splenocytes of C57BL/6 and B10 mice but fail to lyse cells from congenic B10.BR mice. These data indicate linkage of the respective genes to the MHC. Typing of intra $H-2$ recombinant strains showed strongly positive reactions for strains carrying the $H-2D^b$ allele, e.g.,
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Fig. 1. Reactivity of monoclonal anti-Qat-4 and anti-Qat-5 with C56BL/6 splenocytes. (A) Cytotoxicity of ascites fluids in the presence of rabbit complement as determined by dye exclusion. (B) Cytotoxicity of culture supernates.

Table I

| Strain Distribution and Linkage to the H-2 Complex of Antigens Identified by Monoclonal T Antibodies, B16-146 (Anti-Qat-4) and B16-167 (Anti-Qat-5) |
|---|---|---|---|---|---|---|
| Strain | H-2 complex | Titer* of antibody‡ |
| C57BL/6, B10 | k/b | b | b | b | b | 10^-6 | 10^-4 |
| B10 BR | k | k | k | a | b | <1 | <1 |
| B10 A (2R) | k | k/d | b | b | b | 10^-6 | 10^-3 |
| B10 A (4R) | k | k/b | b | b | b | 10^-4 | 10^-4 |
| B10 A (5R) | b | k/d | d | a | b | 10^-3 | <1 |
| B10 HTT | s | s/k | d | c | b | 10^-3 | <1 |
| B10 D2 | d | d | c | c | b | 10^-4 | 10 |
| B10 A | k | k/d | d | a | b | 10^-3 | <1 |
| A BY | b | b | b | b | b | 10^-6 | 10^-2 - 10^-4 |
| C3H SW | b | b | b | c | b | 10^-6 | 10^-4 |
| SJL | s | s | s | a | b | 10^-3 | 10 |
| BALB/c | d | d | c | c | b | <1 | <1 |
| AKR, CBA | k | k | k | b | b | <1 | <1 |

* Average reciprocal 50% titer against lymph node cells obtained in several independent microcytotoxicity experiments.
‡ Ascites fluids were used of either AKD2 mice injected with hybridoma B16-146 or of NMRI nude mice injected with B16-167 hybridoma cells.

B10 A (2R) and B10 A (4R), suggesting linkage of the respective genes to the D end of the H-2 complex. Strains carrying the H-2D^3 allele (B10 D2, B10 A, B10 HTT) react with both antibodies at a much lower titer indicating that H-2^d and H-2^b haplotypes may be associated with different allelic forms of the new antigens.

To locate more precisely the genes for Qat-4 and Qat-5 several H-2/Tla recombinant strains were investigated. The respective gene charts and the results obtained are depicted in Fig. 2. The position of Qa-1 (the only other gene marker in the TL region) and the Qa-1 phenotypes of B6 K1 and B6 K2 are presently in doubt (14) and hence a more precise location of Qat-4 and Qat-5 loci are not possible. In spite of this complication, the positive reaction of both monoclonal antibodies with B6 K2 cells and the absence of reactivity with B6 K1 cells allows a tentative assignment of the respective genes to the Qa-2,3 region. Because of the close linkage to the Qa/Tla region we have provisionally designated the new loci Qat-4 and Qat-5.
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During the course of the mapping studies it was surprising to find that neither A/Boy nor A.Tla b strains reacted with anti-Qat-4 and anti-Qat-5 although both A/J and C57BL/6 were positive. C57BL/6 has been thought to have provided the Qa/Tla region of the A-tla b recombinant. Possible explanations for this observation will be discussed below.

Table II summarizes the results of an extensive strain distribution analysis. Two cytotoxicity procedures were used in parallel. In the first, cytolysis was scored by trypan blue exclusion, and in the other by Cr\textsuperscript{51} release. In most cases strains typing positively for anti-Qat-4 are also positive for anti-Qat-5. Exceptions were strains A/HeJ and A/J, which reacted with the former, but not the latter antibody. In view of the generally much weaker reactions of the anti-Qat-5 antibody it is possible that the assay failed to reveal a weak cross-reactivity. No strain has been found which is positive for Qat-5 and negative for Qat-4.

Selective Reactivity of Monoclonal Anti-Qat-4 and Anti-Qat-5 with Peripheral T-Cell Populations. In view of the observations that anti-Qat-4 and anti-Qat-5 lyse only a proportion of spleen cells (50 and 30%, respectively; see Fig. 1) and because of linkage of the respective genes to the Qa region, T cells were strongly implicated. An analysis was undertaken to verify whether the respective antigens were expressed on either T cells or B cells, or both. The results have confirmed the selective expression on T cells.

As summarized in Table III, thymocytes and anti-Thy-1.2-treated spleen cells (>95% Ia\textsuperscript{+}) did not react with either antibody, whereas nylon-purified T cells (12) and anti-Ig-treated spleen cells (13) (>90% Thy-1.2\textsuperscript{+}) reacted strongly. Anti-Qat-5 killed consistently 35% of peripheral T cells, and anti-Qat-4 was cytolytic for 75% of T cells, with a titer of $10^{-6}$. Thus, both antibodies define two T-cell-associated differentiation antigens which are expressed by distinct subpopulations within peripheral T cells.
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### Table II

**Strain Distribution** of Qat-4 and Qat-5

<table>
<thead>
<tr>
<th>Group</th>
<th>High titered reaction$ (Qat-4* Qat-5*)</th>
<th>Strain Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>B10, C57BL/6, B10.A(2R), B10.A(4R), B6.K2, B6.T/a*, A.BY, C3H.SW, BALB.B, 129, C38, C57L, A.SW</td>
<td></td>
</tr>
<tr>
<td>Group II</td>
<td>Medium titer$ (Qat-4* Qat-5*)</td>
<td>B10.D2, B10.A(5R), B10.HTT, A/J, A/Hej, A/WySn, A.TL, A.TH, SJL, DBA/2</td>
</tr>
<tr>
<td>Group III</td>
<td>Negative (Qat-4- Qat-5-)</td>
<td>B10.BR, B6.K1, B6-H-2*, AKR, CBA/J CBA/HN, C3H/An, C37BR/cdJ, BALB/cBy, A.CA, SEC/1, P/J, BDP, BUB, SWR</td>
</tr>
</tbody>
</table>

* Cytotoxicity obtained with ascites fluids and rabbit complement was determined by trypan blue and \(^{3}C^3\)Cr release.

\$ Higher titer: \(\sim 10^{-6}\) for anti-Qat-4, \(\sim 10^{-3}\) for anti-Qat-5.

\$ Medium titer: \(\sim 10^{-3}\) for anti-Qat-4 and very weak or negative for anti-Qat-5.

### Table III

**Selective Reactivity of Antibodies Anti-Qat-4 and Anti-Qat-5 with Peripheral T Cells from C57BL/6 Mice**

<table>
<thead>
<tr>
<th>Monoclonal antibody*</th>
<th>Specificity</th>
<th>Lysis of:</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Spleen</td>
<td>T cells</td>
<td>B cells</td>
</tr>
<tr>
<td>6/68</td>
<td>Thy-1.2</td>
<td>50</td>
<td>(&gt;95)</td>
<td>0</td>
</tr>
<tr>
<td>B17-263</td>
<td>Ia.8</td>
<td>60</td>
<td>0</td>
<td>(&gt;95)</td>
</tr>
<tr>
<td>B16-146</td>
<td>Qat-4</td>
<td>55</td>
<td>75</td>
<td>0</td>
</tr>
<tr>
<td>B16-167</td>
<td>Qat-5</td>
<td>25</td>
<td>35</td>
<td>0</td>
</tr>
</tbody>
</table>

\* Antibody 6/68 is a monoclonal anti-Thy-1.2 antibody (10). Antibody B17-263 is a monoclonal AKR anti-C57BL/6 antibody with specificity for Ia.8 (G. Hämmerling and H. Lemke, unpublished observations).

\$ T cells were obtained by removal of splenic B cells on rabbit anti-mouse Ig-coated plastic dishes (13). B cells were obtained by treatment of spleen cells with antibody 6/68 (anti-Thy-1.2) plus complement. The average percent lysis determined by several independent microcytotoxicity assays is presented.

Among other tissues, brain, liver, kidney, and erythrocytes were found to be negative as indicated by their inability to absorb the anti-Qat antibodies (data not shown).

**Reactivity with Cells from Nude Mice.** In the course of an analysis of spleen cells of C57BL/6 nu/nu mice as a source of B lymphocytes, it has been found that anti-Qat-4 antibody (but not anti-Qat-5) reacted with a small proportion of cells (see Fig. 3 A). After removal of immunoglobulin-bearing cells (13) from nude spleen the anti-Qat-4-positive cells could be shown to belong to the pool of Ig-negative cells some of which were also Thy-1.2 positive and may therefore constitute T cells (see Fig. 3 B). The failure of anti-Qat-5 to react with C57BL/6 nu/nu spleen cells may again be explained on the basis of the generally weaker cytolytic titer of this antibody as compared to anti-Qat-4. Even this powerful antibody had a titer of only \(10^{-3}\) on C57BL/6 nu/nu target cells, which may point to a low epitope density of Qat-4 determinants on nude spleen cells, as opposed to normal T cells.
Fig. 3. Expression of Qat-4 and Qat-5 on Ig-negative nude spleen cells. (A) Reactivity of ascites fluids of anti-Qat-4 and anti-Qat-5 with unseparated splenocytes from C57BL/6 nu/nu spleens. The anti-Thy-1.2 antibody is derived from culture supernates of hybridoma 6/68 (10). (B) Reactivity with C57BL/6 nu/nu spleen cells depleted of Ig-positive cells by fractionation over anti-Ig-coated plastic dishes (13).

Fig. 4. Fluorescence activated cell sorter analysis of profiles Qat-4 and Qat-5 antigens in C57BL/6 lymph node T cells. T cells were obtained by passage of C57BL/6 lymph node cells over anti-Ig-coated plastic dishes. Indirect fluorescence staining was performed as described in Materials and Methods with ascites fluid at a 1:50 dilution with the exception of the anti-Lyt-2.2 antibody for which undiluted culture supernate of hybridoma 19/178 was used. The anti-Thy-1.2 antibody was obtained from NMRI nu/nu mice bearing the Thy-1.2 specific hybridoma 6/68 (10) in ascites form, while the negative control consisted of ascites fluid from BALB/c mice bearing the plasmacytoma P3-X63-Ag8.

**Fluorescence Profile of T Cells Reactive with Anti-Qat-4 and Anti-Qat-5.** Additional information on the reactivity pattern of anti-Qat-4 and anti-Qat-5 was obtained through analysis by a fluorescence-activated cell sorter (FACS). B cells were depleted from C57BL/6 lymph node cells by adherence to rabbit anti-mouse-Ig-coated dishes (13), and the resulting purified T cells were reacted with ascites fluids at a 1:50 dilution, and after washing, with fluorescein-conjugated rabbit anti-mouse Ig. As a negative control a 1:50 dilution of ascites fluid of BALB/c mice bearing the plasmacytoma P3-X63-Ag8 was used, although positive controls consisted of monoclonal anti-Thy-1.2 of clone 5/68, which like anti-Qat-4 and anti-Qat-5 belongs to the kappa/μ class (10). Another positive control was culture supernatant from monoclonal anti-Ly-2.2 antibody hybridoma 19/178 (kappa, IgG2A) (U. Hämmerling, unpublished observations). The fluorescence profiles of Qat-4 obtained with the FACS (see Fig. 4) display a distinct shoulder which comprised 68.5% of peripheral T cells. For Qat-5 the number of positive cells was calculated to be 29.2% above the background control with P3-X63-Ag8 antibody. However, no distinct peak or shoulder was found.
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(see Fig. 4A). The cells displaying a continuum of weakly positive to negative, made the exact determination of positive cells somewhat ambiguous. However, because cytotoxicity analysis and FACS analysis were in close agreement for practical purposes, a distribution of 70% for Qat-4 and 30% of Qat-5 within the peripheral T population may be sufficient. For comparison, the fluorescence profiles of the same T cells stained with monoclonal anti-Ly-2.2 and anti-Thy-1.2 are also presented (see Fig. 4B). Staining with anti-Ly-2.2 results in a sharp peak comprising T cells, whereas staining with anti-Thy-1.2 covers a wide range from dim to very bright cells. Although these data do not allow firm calculations of the number of antigenic determinants per cell, it can be estimated that on the average, T cells express several times more Thy-1.2 antigens than Qat-4 and Qat-5 determinants.

Discussion

The availability of techniques to produce monoclonal antibodies against specific alloantigenic determinants provided us with the opportunity to search for antibodies against new cell surface structures in immunizations between noncongenic partner strains such as AKR anti-C57BL/6. Of the several monoclonal antibodies produced in this immunization, two, B16-146 and B16-167, appear to recognize new antigenic determinants governed by the Qa region. Because these new antigens are selectively expressed on subpopulations of peripheral T lymphocytes, they were provisionally designated Qat-4 and Qat-5, respectively. The loci (or locus) governing the expression of these antigenic determinants were located on the basis of serological studies of H-2 and Tla region recombinants and congenic strains. The more salient features of this analysis are given below:

(a) The strain distribution of Qat-4 and Qat-5 according to direct cytotoxicity assays on nylon column purified cells and lymph node lymphocytes indicates that Qat-4 and Qat-5 expression are governed by genetic loci on chromosome 17 which are different from H-2, la, and Tla.

(b) Reactivity of these antisera against B6.K2 cells and not B6.K1 cells indicates that the locus or loci governing Qat-4 and Qat-5 are near Qa-2 and Qa-3 but not identical to these loci on the basis of tissue distributions (see below) and on the basis of strain distributions (Table II).

(c) The expression of Qat-4 and Qat-5 seems to depend on the expression of Qa-2. All strains which are Qa-2- are also Qat-4- and Qat-5-, whereas Qa-2+ strains can either be Qat-4+ Qat-5+, Qat-4+ Qat-5-, or Qat-4- Qat-5-. No Qat-4- Qat-5+ strain has yet been detected.

(d) Qat-4+ strains which possess the H-2Dd allele react to anti-Qat-4 with a much lower titer in the direct cytotoxicity test than H-2Db strains, suggesting that H-2Dd strains may bear slightly different Qat-4 antigens than H-2Db strains (Table I).

Studies on the tissue distribution show clearly that among lymphocytes, Qat-4 and Qat-5 antigens are restricted to peripheral T lymphocytes on which they occur in a lower density than Thy-1 determinants, as indicated by fluorescence studies. They are absent from thymocytes, suggesting that immature, Qat-4 negative thymocytes differentiate into Qat-4 positive T cells. However, Qat-4 antigen is also present, albeit in a low density, on Ig-negative lymphocytes from nude mice, indicating that these cells can acquire a T-cell differentiation marker in the absence of a thymic environment. Similar observations have been reported by Loor and Roelants (15) with regard to...
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the expression of Thy-1.2 on spleen cells in nude mice.

It is not clear whether Qat-4 and Qat-5 antigens are identical with or different from any of the known antigens specified by the Tla region. Of these, TL, Qa-1, and Qa-2 can be excluded because these antigens are expressed on thymocytes which lack Qat-4 and Qat-5. In contrast, Qa-3, Qat-4, and Qat-5 antigens appear to be similar with regard to their restricted expression on peripheral T cells. However, the strain distributions of known Qa-3 alleles (14) and of Qat-4 and Qat-5 are not completely identical. Qa-3 can be found on BALB/cJ cells (5) which are unreactive with our monoclonal antibodies. Qa antigens defined with conventional antisera, may still represent complex systems and may include Qat-4 and Qat-5 antigens. Obviously, highly specific monoclonal antibodies to Qa antigens and TL being developed in our laboratories will be more suitable reagents for the definition of antigenic determinants encoded by the Tla region.

The observation that the two monoclonal antibodies react with a different percentage of T lymphocytes (70 and 30%, respectively) does not necessarily distinguish the Qat-4 and Qat-5 antigens as two different antigens. They may represent two different antigenic determinants on the same molecule. However, if this were the case then one has to assume that the two determinants are located far apart on the Qat molecule because the binding of radioiodinated anti-Qat-5 to T cells could only be inhibited by preincubation with cold anti-Qat-5 but not by cold anti-Qat-4 (unpublished data).

Depletion of lymphocyte subpopulations with anti-Qat sera should reveal if these antibodies can be used for the definition of functional T-cell populations. Preliminary studies indicate that treatment of T helper cells with either anti-Qat-4 or anti-Qat-5 and complement had no effect on helper activity for antibody formation, whereas with anti-Qat-5 an increase in helper activity was frequently observed, suggesting the removal of regulatory T cells by this antibody.

Summary

Two new lymphocyte antigens, provisionally designated Qat-4 and Qat-5 have been identified with two different hybridoma-derived, monoclonal AKR anti-C57BL/6 antibodies. These antigens are governed by genes located to the right (distal) end of the H-2 complex, within the Qa-2,3 region. Qat-4 and Qat-5 antigens which do not seem to be identical with Qa-2,3 or TL antigens are absent from Ig+ lymphocytes and thymocytes. They are only present on a fraction of peripheral T cells. Thus, Qat-4 is expressed on 70%, and Qat-5 on 30% of splenic and lymph node T cells, Qat-4 is also found on the majority of Ig- cells from athymic nude mice. These findings illustrate the complexity of the chromosome segment between the H-2D and Tla loci and they emphasize the role of major histocompatibility complex-associated genes for the differentiation of T cells into different subpopulations with possibly distinct immunologic functions.

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