ABSTRACT

For the further characterization of bovine leukemia virus (BLV)-induced leukemogenesis, we investigated the association between polymorphism of ovine leukocyte antigen (OLA)-DRB1 gene and tumor development after infection of sheep with BLV. We infected 28 sheep with BLV and cloned exon 2 of the OLA-DRB1 gene from asymptomatic animals and from animals with lymphoma. Sequence analysis revealed that, among 12 healthy sheep without any evidence of tumor, ten (83.3%) carried DRB1 alleles encoding Arg-Lys (RK) at positions β70/71 as compared with only 6 (37.5%) of the 16 sheep with lymphoma, which suggested that alleles encoding the RK motif might protect against development of tumors after infection by BLV. By contrast, alleles encoding Ser-Arg (SR) at positions β70/71 were present at a significantly elevated frequency in sheep with lymphoma as compared with the healthy carriers, which indicated that OLA-DRB1 alleles encoding the SR motif might be positively related to susceptibility to tumor development. The two amino acids in these motifs line a pocket that accommodates the side chain of a bound peptide according to a model of the crystal structure of human leukocyte antigen (HLA)-DR1. To analyze immunoreactions of sheep with alleles that encoded RK or SR at β70/71, we selected sheep with either the RK/SR genotypes or the SR/SR genotypes and immunized them with a mixture of multiple synthetic antigenic peptides that corresponded to T-helper, T-cytotoxic, and B-cell epitopes of the BLV envelope glycoprotein gp51. Two weeks after the last immunization, all of the sheep were challenged with BLV. Sheep with the RK/SR genotype produced neutralizing antibodies against BLV; they eliminated BLV completely within 28 weeks of the BLV challenge, and they gave strong lymphocyte-proliferative responses to the peptides used for immunization. Moreover, such animals did not develop lymphoma. By contrast, sheep with the SR/SR genotype continued to produce BLV throughout the experimental period and developed terminal disease. Our results indicate that the differences in immunoresponses were due to differences in major histocompatibility complex class II alleles and reflected the risk of BLV-induced leukemogenesis. In addition, it appears that susceptibility to tumor development may be determined to some extent by polymorphic residues binding to antigenic peptides directly within the binding cleft of the OLA-DR molecule.

INTRODUCTION

BLV1 and HTLV-I and II constitute a unique subgroup within the retrovirus family that is characterized by distinct genetic content, genomic organization, and strategy for gene expression (1, 2). Replication of these viruses is not only regulated by the classical structural genes of retroviruses but is also regulated at the transcriptional level (3, 4) and the posttranscriptional level (5, 6) by Tax and Rex, respectively, which are products of their own genes. BLV is associated with enzootic bovine leukosis, which is the most common neoplastic disease of cattle. Infection by BLV can remain clinically silent, with the host in an aleukemic state, or it can emerge as PL, characterized by an increased number of normal B-lymphocytes and, more rarely, as B-cell lymphoma in various lymph nodes after a long latent period (1). Under experimental conditions, sheep can easily be infected with BLV and tend to develop B-cell leukemia/lymphoma at higher frequencies and after shorter latent periods than cattle (7, 8). In addition, lymphocytosis very often indicates the onset of the tumor phase. It is of interest that the transformed phenotype of the B lymphocytes that are the target of BLV is CD5− in sheep (9−11), whereas it is CD5+ in cattle (11, 12). Thus, ovine leukemia may constitute a good experimental model for understanding the virus-induced leukemias of the BLV-HTLV group.

The pathogenesis of infections clearly involves immunoregulatory host factors, which include products of the MHC. The HLA molecules are glycoproteins on the surface of receptor cells that bind peptides and present them to T cells (13, 14). This interaction causes stimulation of T cells and activation of an immune response. Class I and class II HLA molecules have different domain organization but similar structure (15, 16). Polymorphic residues in both class I and class II molecules are clustered within the peptide-binding region and are responsible for the different peptide specificities of different histocompatibility molecules. Both class I and class II molecules have allele-specific binding motifs (17, 18). Peptides bound by class I molecules are of defined length (8−10 residues; Ref. 19). Peptides bound by class II molecules are longer with no apparent restrictions with respect to peptide length (20, 21). The main-chain atoms of the peptides form hydrogen bonds with HLA residues that are conserved in most class II molecules, and the side chains are accommodated by polymorphic pockets at the binding site. These pockets seem to determine the peptide specificity of different class II proteins (22). Thus, polymorphic residues in the binding cleft for HLA class II molecules control the binding of foreign peptides and also, indirectly, the immune response to these peptides. Moreover, nucleotide sequence polymorphism in the genes for HLA class II molecules determines the specificity of the immune response and plays a role in conferring resistance or susceptibility to: (a) chronic autoimmune disorders such as rheumatoid arthritis, insulin-dependent diabetes mellitus, pemphigus vulgaris, and multiple sclerosis (23); (b) infectious diseases such as tuberculosis leprosy (24) and malaria (25); and (c) malignancies such as carcinoma (26−28) and melanoma (29).

In BLV, the genes for BoLA seem to be important genetic determinants of resistance or sensitivity to subclinical progression of BLV infection (11). The influence of BoLA polymorphism on the development of lymphocytosis was first indicated in analyses of different BoLA-A alleles in different races of the cattle (30−32). Subsequently, BoLA class II genes were shown to be more strongly associated with resistance and susceptibility to PL than the BoLA-A locus. Among BoLA class II genes, BoLA-DRB3 functional genes, which are highly polymorphic, were found to have a stronger association with suscep-
tibility to PL than DRB2 or DQB (33–35). Nucleotide sequence analysis of the second exon of these DRB3 alleles revealed the presence of codons for specific amino acids at positions 70–71 of the β1 domain exclusively in alleles associated with resistance to PL (34). Thus, these results pointed strongly toward the involvement of BoLA-DRB3 in the subclinical progression of BLV infection. However, the association between the development of cancer and the BoLA complex remains unknown. Therefore, to investigate the association between MHC class II genes and BLV-induced leukemogenesis, we chose to study sheep, which provide a useful experimental model for infection by BLV and which carry MHC class II genes that are very similar to genes for BoLA (36–40). We identified OLA-DRB1 alleles (corresponding to the BoLA-DRB3) that were associated with resistance and with susceptibility to BLV-induced tumorigenesis. Furthermore, to investigate the role of the OLA system in tumor development, we examined the responses to vaccination with specific peptides and a subsequent challenge with BLV in sheep that carried resistance or susceptibility alleles.

MATERIALS AND METHODS

Animals—Inoculation with BLV and Vaccination with Peptide. Sheep (Suffolk, Corridale, and crossbred) of 0.5–5 years of age, which were negative for BLV-specific antibodies, were provided by the Takikawa Animal Husbandry Experiment Station (Takikawa, Japan) and divided into two groups. To analyze the relationship between the progression of BLV-induced ovine lymphoma and polymorphism of OLA-DRB1 alleles, we inoculated the 28 sheep s.c. with 1.0 ml of blood from BLV-infected cattle. Then we classified the sheep into two types according to established criteria (8) and the pattern of integration of the BLV provirus into the host genome, as follows: (a) BLV-infected but clinically normal sheep, and (b) sheep with lymphoma (Table 1). Three additional animals were used to investigate the effects of peptide vaccination against a challenge with BLV (Table 6). Two sheep were immunized with a mixture of synthetic antigenic peptides, namely 0.5 mg each of: (a) Th (PGQRRFGRGAMYTVYDCE; BLV Env 61–78); (b) Tc (GIFTLTYDWMAY); and SRB3, 5′-CTCTCTCTGCAGCACATTTCCT-3′, and SRB3, 5′-AAGTCGACCGCTG-3′, and 2.5 units of Tφq polymerase had been added. The conditions for PCR were an initial 4-min incubation at 94°C, which was followed by 35 cycles of 94°C for 30 s, 61°C for 30 s, and a final extension at 72°C for 10 min. Then, 5 μl of PCR products were subjected to electrophoresis in a 3% agarose gel. Ten μl of each reaction mixture after PCR that contained a fragment of the expected size (373 bp) were then digested for 2 h at 37°C with Rsd in a total volume of 20 μl, and then this mixture was fractionated by electrophoresis in a 3% agarose gel. Clones that yielded two different patterns of fragment per animal were selected, and the nucleotide sequencing of both strands of each allele was determined by the dyeoxy chain-termination method (47) with a BcaBEST sequence kit (Takara Shuzo, Otsu, Japan). The predicted amino acid sequences were analyzed using the program package from the Wisconsin Genetics Computer Group (48).

Statistical Methods. Depending on the sample size, Fisher’s exact probability test or the χ² test was used to analyze the results. Significance was accepted at 0.05 for all of the tests.

Other Procedures. Assays for neutralizing antibody, lymphocyte proliferation, and syncytium formation were performed as described previously (49). For detection of BLV provirus, genomic DNA was completely digested with either SacI or HindIII and analyzed by Southern blotting (50) with, as a probe, the 32P-labeled 6.9-kilobase EcoRI/SalI fragment of an infectious full-length molecular clone of BLV, pBLV-IF (51). BLV provirus integrated into the host’s genome was amplified from chromosomal DNA (100 ng to 1 mg) by PCR with two sets of primers: (a) 7192 and 533; and (b) 7192 and 8580 (52). PCR products were subjected to electrophoresis and then analyzed by Southern blotting as described above. For detection of antibodies against BLV, an immunodiffusion test was performed with BLV gp51 and internal protein 24 as antigens.

RESULTS

Isolation and Identification of OLA-DRB1 Alleles from BLV-infected Sheep. To analyze the association between polymorphism of the OLA-DRB1 gene and tumor development after infection with BLV, we inoculated 28 sheep with 1 ml of whole blood from BLV-infected cattle. After inoculation, we were able to distinguish between sheep that showed evidence of asymptomatic and lymphoma stages of progression of BLV-induced lymphomagenesis (Table 1). Sixteen sheep developed systemic lymphoma between 2 and 6 years after infection, and several had leukemia, with an increase in the number of atypical mononuclear cells in the peripheral blood. Tumors from these animals seemed to be the result of monoclonal expansion of single cells that each carried only one copy of the viral genome, as determined by genomic Southern analysis with HindIII, which produces two fragments of the viral genome per integrated copy of BLV. By contrast, no abnormal clinical and hematological parameters were detected in the remaining 12 sheep during the period of the experiment. Nine of these latter animals gave a smeared band of fragments of 5 to 20 kb on genomic Southern analysis with HindIII, which indicated that the PBMC from these animals consisted of polyclonal populations of cells that carried multiple BLV proviruses. In 3 sheep (pr2445, pr2423, and pr2260), we detected integrated BLV viruses by PCR (a more sensitive method for detecting proviral genomes than genomic Southern analysis), which suggested that the population of BLV-carrying lymphocytes circulating in the blood was very small. Thus, we classified the 28 BLV-infected sheep into two populations: the 12 BLV-infected but healthy sheep and the 16 sheep with lymphoma.
We cloned exon 2 of *OLA-DRB1* alleles from the sheep in the two populations and determined the nucleotide sequences of both strands from each clone. First, we isolated two distinct *OLA-DRB1* alleles from each individual as follows: (a) before sequencing, the PCR-RFLP method was used to identify the cloned allele as one of the two possible OLA haplotypes in each donor; and (b) after DNA sequencing, the sequences of the cloned alleles from an individual were checked against the known PCR-RFLP genotype of the donor animals. The DNA sequences of all of the alleles contained the restriction sites predicted from the PCR-RFLP genotype (data not shown). Next, we compared the deduced amino acid sequence of the β1 domain encoded by these alleles with those sequences encoded by 44 previously reported *OLA-DRB1* alleles (53–55). We identified 18 distinct alleles in the 28 sheep, and 10 of these 18 were different from the previously characterized *OLA-DRB1* alleles (Tables 1 and 2).

**OLA-DRB1** Alleles Associated with Resistance and Susceptibility to BLV-induced Leukemogenesis. The numbers of individuals that were positive for each of the different *OLA-DRB1* alleles in sheep at the asymptomatic stage and the lymphoma stage are shown in Table 2. The proportion of healthy sheep positive for *OLA-DRB1*<sup>*n*3</sup> [9 (37.5%) of 24] was higher than the proportion-bearing individuals with lymphoma that were positive for the same allele [3 (9.4%) of 32; *P* = 0.0195]. No other alleles were associated with a statistically significant difference between the two populations. This result suggested the presence of epitope(s) encoded by the *OLA-DRB1*<sup>*n*3</sup> sequence that showed important for disease resistance. To identify the amino acid residues that were associated with sensitivity to BLV-induced ovine lymphoma, we aligned the residues that differed among the *OLA-DRB1* alleles that were positively and negatively associated with tumor progression (Fig. 1). The *OLA-DRB1*<sup>*n*3</sup> allele was found most frequently in healthy sheep and appeared to encode amino acids RK at positions 70/71. Both residues are in an α-helical region and point into the antigen-binding groove in the predicted model of the class II β chain (22). We recalled that BoLA-DRB3 alleles that are characterized by the Glu-Arg motif at positions 70/71 are associated with resistance to the development of BLV-induced PL (34). Therefore, we reexamined whether these amino acid residues might be correlated with resistance and susceptibility to lymphoma (Table 3). Among healthy sheep, 87.5% carried alleles that encoded Arg at position 70 as compared with 40.6% in the lymphoma group.
Ser and Gln at position 70 in the same population, alleles encoding non-Arg amino acids such as Arg and Thr, and alleles encoding other motifs such as RR, were present at a significantly higher frequency in lymphoma cases as compared with healthy sheep (lymphoma, 37.5%; healthy, 8.3%; P = 0.0146). These results demonstrated that OLA-DRB1 alleles that encoded the SR motif at positions β70/71 were positively related to tumor development after infection by BLV.

We compared frequencies of OLA-DRB1 genotypes at the two stages of the disease (Table 4). The RK/RK genotype was found only in healthy sheep (P = 0.0025), a result that supports the hypothesis that the RK/RK genotype is actually associated with resistance to lymphoma. By contrast, the positive association of the SR/SR genotype with susceptibility to lymphoma was significant at the 100% level. In the case of RK/SR, RK/QT, RK/RR and SR/RR genotypes, the differences between populations of sheep were not significant. Resistance, susceptibility to BLV-induced ovine lymphoma did not seem to be a dominant trait.

Experimental Infection with BLV of Sheep with the Resistance-associated RK Motif or the Susceptibility-associated SR Motif. To investigate the role of the OLA system in tumor development, we analyzed the immunoresponses of sheep that carried resistance and susceptibility alleles. We selected sheep that had the RK/SR or SR/SR genotype and immunized them with a mixture of multiple synthetic antigenic peptides (Th, Tc, and B epitopes of BLV envelope glycoprotein gp51). Two weeks after the last immunization, all of the sheep were challenged with BLV (Fig. 2 and Table 6). In the sheep with the RK/SR genotype, BLV-neutralizing activity increased significantly after the third immunization but decreased gradually after the BLV challenge (Fig. 2B). In contrast, the sheep with the SR/SR genotype did not produce neutralizing antibodies immediately after immunization with the peptides. To demonstrate the spread of BLV infection, we titrated BLV by the syncytium assay for 1–40 weeks after the BLV challenge (Fig. 2A). During the first 2 weeks, BLV was isolated from all of the sheep. However, in the sheep with the RK/SR genotype, the titer of BLV started to decrease markedly at 4 weeks after the BLV challenge. At 28 weeks, hardly any BLV could be isolated. In the sheep with the SR/SR genotype as well as in control sheep inoculated with PBS, the titer of BLV reached a peak 4 weeks after the BLV challenge.

Table 3. Association of DRB1 epitopes with BLV-induced ovine lymphoma

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Lympoma (n = 32)</th>
<th>Healthy (n = 24)</th>
<th>( \beta^{70} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>R(^{70})</td>
<td>13</td>
<td>40.6</td>
<td>21</td>
</tr>
<tr>
<td>Non-R(^{70})</td>
<td>19</td>
<td>59.4</td>
<td>3</td>
</tr>
<tr>
<td>S(^{70})</td>
<td>12</td>
<td>37.5</td>
<td>2</td>
</tr>
<tr>
<td>Q(^{70})</td>
<td>7</td>
<td>21.9</td>
<td>1</td>
</tr>
<tr>
<td>K(^{71})</td>
<td>7</td>
<td>21.9</td>
<td>16</td>
</tr>
<tr>
<td>Non-K(^{71})</td>
<td>25</td>
<td>78.1</td>
<td>8</td>
</tr>
<tr>
<td>R(^{71})</td>
<td>19</td>
<td>59.4</td>
<td>2</td>
</tr>
<tr>
<td>T(^{71})</td>
<td>6</td>
<td>21.4</td>
<td>1</td>
</tr>
<tr>
<td>R(^{70})K(^{71})</td>
<td>6</td>
<td>18.8</td>
<td>16</td>
</tr>
<tr>
<td>R(^{70})Q(^{71})</td>
<td>7</td>
<td>37.5</td>
<td>5</td>
</tr>
<tr>
<td>Q(^{70})K(^{71})</td>
<td>1</td>
<td>3.1</td>
<td>0</td>
</tr>
<tr>
<td>Non-R(^{70}) , non-K(^{71})</td>
<td>18</td>
<td>66.3</td>
<td>3</td>
</tr>
<tr>
<td>S(^{70})Q(^{71})</td>
<td>12</td>
<td>37.5</td>
<td>2</td>
</tr>
<tr>
<td>Q(^{70})T(^{71})</td>
<td>6</td>
<td>18.8</td>
<td>1</td>
</tr>
</tbody>
</table>

* Fisher’s exact test was used to determine statistical significance.

* Number of alleles positive that encoded a particular epitope at position \( \beta^{70} \) and/or \( \beta^{71} \).

(P = 0.0007). Moreover, 66.7% of healthy sheep carried alleles that encoded Lys at position \( \beta^{71} \) as compared with 21.9% in the lymphoma group (P = 0.0010). Likewise, alleles characterized by the RK motif at positions \( \beta^{70/71} \) were present at a significantly higher frequency in healthy sheep as compared with sheep with lymphoma (healthy, 66.7%; lymphoma, 18.8%; P = 0.0004). As shown in Fig. 1, those alleles included OLA-DRB1*n19, OLA-DRB1*n10, OLA-DRB1*n11, OLA-DRB1*n23, OLA-DRB1*n24, and OLA-DRB1*n25. By contrast, in the same population, alleles encoding non-Arg amino acids such as Ser and Gln at position \( \beta^{70} \), alleles encoding non-Lys amino acids such as Arg and Thr, and alleles encoding other motifs such as RR, QK, SR, and QT at positions \( \beta^{70/71} \) were present at significantly lower frequencies. These results suggested that OLA-DRB1 alleles that encode the RK motif at positions \( \beta^{70/71} \) may have a strong protective effect against tumor development after infection by BLV.

We next identified the OLA-DRB1 alleles that were positively associated with susceptibility to BLV-induced ovine leukemogenesis (Table 3). Among sheep with lymphoma, 59.4% carried alleles encoding non-Arg amino acids at position \( \beta^{70} \) as compared with 12.5% in the healthy group (P = 0.0007); 78.1% carried alleles encoding non-Lys amino acids at position \( \beta^{71} \) as compared with 33.3% in the healthy group (P = 0.0007); and 66.3% carried alleles for non-Arg and non-Lys amino acids at positions \( \beta^{70/71} \) as compared with 12.5% in the healthy group (P = 0.0009). However, only alleles encoding Ser and Gln at position \( \beta^{70} \) and Arg at position \( \beta^{71} \) were found at significantly higher frequencies than in the healthy controls. Accordingly, alleles characterized by the SR motif but not QT, QK nor RR at positions \( \beta^{70/71} \) in lymphoma cases were present in a statistically significant elevated frequency in lymphoma cases as compared with healthy sheep (lymphoma, 37.5%; healthy, 8.3%; P = 0.0146). These results demonstrated that OLA-DRB1 alleles that encoded the SR motif at positions \( \beta^{70/71} \) were positively related to tumor development after infection by BLV.

Table 4. Association between BLV-induced ovine lymphoma and amino acid motifs at positions \( \beta^{70/71} \)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Lympoma (n = 16)</th>
<th>Healthy (n = 12)</th>
<th>( \beta^{70/71} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>RK/RK</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>SR/SR</td>
<td>4</td>
<td>25.0</td>
<td>0</td>
</tr>
<tr>
<td>RK/QT</td>
<td>3</td>
<td>18.8</td>
<td>2</td>
</tr>
<tr>
<td>RK/RR</td>
<td>1</td>
<td>6.3</td>
<td>1</td>
</tr>
<tr>
<td>QK/QT</td>
<td>1</td>
<td>6.3</td>
<td>0</td>
</tr>
<tr>
<td>SR/RR</td>
<td>1</td>
<td>6.3</td>
<td>0</td>
</tr>
<tr>
<td>QT/RR</td>
<td>2</td>
<td>12.5</td>
<td>0</td>
</tr>
<tr>
<td>RR/RR</td>
<td>2</td>
<td>12.5</td>
<td>2</td>
</tr>
</tbody>
</table>

* Fisher’s exact test was used to determine statistical significance.

* Number of individual animals with a particular motif at positions \( \beta^{70/71} \).
the sheep with the RK/SR genotype was higher than that from the sheep with the SR/SR genotype. Furthermore, the sheep with the RK/SR genotype gave a strong response to the Th peptide and the mixture of peptides 2 weeks after the BLV challenge. Therefore, as indicated in Table 6, the sheep with the resistance-associated RK motif, which induced significant production of neutralizing antibodies against BLV and considerable lymphocyte proliferation in response to the peptides, eliminated BLV and did not develop lymphoma. In contrast, the sheep with the susceptibility-associated SR/SR genotype developed terminal disease.

**DISCUSSION**

In this study, we sequenced OLA-DRB1 alleles of sheep experimentally infected with BLV and demonstrated the existence of alleles associated with resistance and susceptibility to BLV-induced leukemogenesis. We confirmed our results by showing that sheep with resistance alleles did not develop leukemia after vaccination with specific peptides and a subsequent challenge with BLV, whereas the sheep with susceptibility alleles developed lymphoma. A previous study (34) revealed strong linkage between BoLA-DRB3 and sensitivity to subclinical progression of BLV infection. However, the association of cancer development with the BoLA complex remains unknown. Therefore, to our knowledge, this is the first report that tumor development caused by BLV is linked to polymorphism of MHC class II genes. Furthermore, our present results clearly showed that quantitative and/or qualitative aspects of immunoresponses such as lymphocyte proliferation in response to the peptides, production of neutralizing antibodies against BLV, and elimination of BLV depended on the particular allelic forms of the MHC class II molecules expressed by an individual and, in particular, on certain polymorphic amino acid residues in class II molecules. From these data, we propose that leukemogenesis induced by BLV requires a genetic factor in the host that is associated with the immune response. Thus,

![Fig. 2. Virus titer and neutralizing activities in BLV-challenged sheep. Sheep with either the RK/SR or the SR/SR genotype were immunized three times with a mixture of synthetic Th-, Tc-, and B-epitope peptides (B-arrows). Two weeks after the final immunization, all of the sheep were challenged with BLV (Ch-arrow). Sheep with the RK/SR genotype were inoculated with PBS instead of peptides as a control. Two weeks after the last immunization, all three of the sheep were productively infected with BLV and indicator cc81 cells (5 × 10^5) were tested for their ability to produce BLV during coculture with cc81 cells, a cat cell line transformed by mouse sarcoma virus, (1 × 10^5) indicator cells in 24-well plates for 5–6 days. The cells were then fixed with 25% (v/v) acetic acid in methanol for 5 min and stained with 10% Giemsa solution for 30 min. Cells containing more than five nuclei were counted as syncytia. Bars represent standard deviations (SD) of mean for triplicated determinations.](image)

**Table 5** Lymphocyte proliferative responses of PBMC from immunized sheep to peptide antigens

PBMC were collected from sheep with the RK/SR or SR/SR genotype 1 week after the final immunization and 2 weeks after challenge with BLV. These cells were cultured in triplicate wells (5 × 10^5 cells/well) of a 96-well microplate with 100 μg/ml Th, Tc, or a mixture of the two peptides for 5 days. Sixteen h before cells were harvested, 1.0 μCi of [3H]thymidine was added to each well. Cells were harvested on glass-fiber filters, and the incorporation of [3H]thymidine into DNA was determined with a liquid scintillation counter.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Th</th>
<th>Tc</th>
<th>Th + Tc</th>
<th>S.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>RK/SR</td>
<td>1.91 ± 0.50</td>
<td>1.61 ± 0.07</td>
<td>2.16 ± 0.45</td>
<td>1.09 ± 0.26</td>
</tr>
<tr>
<td>SR/SR</td>
<td>1.09 ± 0.26</td>
<td>0.99 ± 0.11</td>
<td>1.22 ± 0.35</td>
<td>0.65 ± 0.13</td>
</tr>
<tr>
<td>RK/SR (control)</td>
<td>0.67 ± 0.19</td>
<td>0.62 ± 0.06</td>
<td>2.26 ± 0.06</td>
<td>1.27 ± 0.15</td>
</tr>
<tr>
<td>SR/SR (control)</td>
<td>0.99 ± 0.11</td>
<td>1.22 ± 0.35</td>
<td>2.09 ± 0.10</td>
<td>0.62 ± 0.06</td>
</tr>
</tbody>
</table>

**Table 6** Summary of immunoresponses to a challenge with BLV after immunization

Three male 4- to 5-month-old sheep (Suffolk breed) were used. Sheep with either the RK/SR or SR/SR genotype were immunized with a mixture of synthetic peptides and then challenged with BLV. A sheep with the RK/SR genotype was inoculated with PBS instead of peptides as a control. Two weeks after the last immunization, all three of the sheep were challenged with BLV. Responses were assessed as described previously (49).

<table>
<thead>
<tr>
<th>Test</th>
<th>RK/SR</th>
<th>SR/SR</th>
<th>RK/SR (control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutralizing activity after immunization</td>
<td>↑</td>
<td>→</td>
<td>→</td>
</tr>
<tr>
<td>Virus titer</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Lymphocyte proliferative response</td>
<td>↑</td>
<td>→</td>
<td>→</td>
</tr>
<tr>
<td>Progression of disease after challenge</td>
<td>Health</td>
<td>Lymphoma</td>
<td>Health</td>
</tr>
</tbody>
</table>

* Changes in neutralizing titer, virus titer, and the lymphocyte proliferative response are indicated as follows: ↑, increase; ↓, decrease; →, no change.

* S.I. =

\[ \frac{cpm \text{ in the stimulated culture}}{cpm \text{ in the unstimulated control culture}} \]
sheep seem to provide a good model for studies of the underlying mechanisms by which polymorphism in MHC class II genes determines the specificity of a host’s immune system or is correlated with tumor development.

Our findings suggest that the alleles of the OLA-DRB1 gene that encode the RK motif and the SR motif at positions β70/71 are associated with resistance and susceptibility, respectively, to the development of BLV-induced lymphoma. Xu et al. (34) similarly reported the presence of the amino acids Glu-Arg at positions 70 and 71 of the DRβ chain in BoLA haplotypes associated with resistance to PL in cattle. What is the significance of the presence of RK or SR at amino acid positions β70/71? We respond: the amino acid residues 70–71 of the HLA-DRB1 domain from peptide-binding pocket 4 (22). The amino acids at positions β70/71 may have a dramatic effect on responses of T cells. From studies of transfectants that expressed wild-type or mutant HLA-DR molecules with single amino acid substitutions, Fu et al. (56) proposed that the DRβ residues 13, 70, 71, 74, and 78, which are located in peptide-binding pocket 4, may exert a major and disproportional influence on the outcome of T-cell recognition as compared with other polymorphic residues. Indeed, as indicated in Table 6, sheep with the resistance-associated RK motif at positions β70/71 produced significant amounts of neutralization antibodies against BLV and a high level of lymphocyte proliferation in response to specific peptides, whereas the sheep with the susceptibility-associated SR/SR motif had weak immune responses. An alternative hypothesis is that the substitution of two amino acids may influence the kind of peptide that can be accommodated in the peptide-binding pocket. In tuberculoid leprosy, disease-associated alleles were characterized by a binding motif that included a positively charged Arg residue at position β70/71 (24). Similarly, a major change in charge from a positively charged Lys residue to a negatively charged Glu residue at position 69 of the DPβ chain, which corresponds to position 71 of the DRβ chain, seems to be associated with susceptibility to chronic beryllium disease, a lung disorder related to beryllium exposure (57). Moreover, alleles that are positively associated with rheumatoid arthritis encode Lys at position 71 of the HLA-DRβ chain and bind peptides with a negative charge in peptide-binding pocket 4. The reverse is true for alleles that are negatively associated with rheumatoid arthritis. They encode Glu at this position, and peptides with a positive charge are bound by pocket 4. Such evidence suggests that residue 71 in the HLA-DRβ chain may be a major predictor of rheumatoid arthritis (58).

In the present study, the resistance-associated RK motif represents a positive charge, whereas the susceptibility-associated SR motif does not represent negatively or positively charged amino acids. The molecular mechanism of the association between BLV-induced leukemogenesis and MHC polymorphism is unclear. However, it is possible that the alleles that encode the RK motif and the SR motif, respectively, at positions β70/71 have differing effects via their involvement in the binding of a foreign peptide and activation of an initial immune response to BLV infection that depends on the particular allele. In this way, each could influence the susceptibility to tumor development.

Our result that different OLA-DRB1 alleles are associated with different risks of developing leukemia is supported by the following earlier findings about the association of the development of certain cancers with HLA class II genes. Özdemir et al. (26) reported a relationship between certain HLA-DRB1 alleles and the malignancy of renal cell carcinoma. There are also some reports of an association between the incidence of cervical carcinoma and of melanoma with HLA-DQ genes (27, 29, 59). Moreover, there appears to be an association between the HLA-DQw3 antigen and susceptibility to development of a cervical cancer, which is known to be aetologically associated with human papilloma virus (27, 59). In addition, Han et al. (60) reported that both the regression and malignant conversion of viral papillomas in rabbits are linked to the MHC class II genes. However, many women infected with human papilloma viruses do not develop cervical cancer; and, thus, a multiple-hit model has been proposed to explain the development of this cancer (61). In the case of cancers that occur after a long latent period with other factors being involved in the development process, the HLA haplotype may be important as a prognostic factor. Likewise, infection by BLV is probably not sufficient for leukemogenesis. Some additional rare event, for example, a mutation in the p53 tumor-suppressor gene, must be involved in the leukemogenic process (62). Thus, class II genes may be the most useful markers and may be important host-related immunogenetic factors in the susceptibility to several virus-induced tumors. Possible environmental factors and other susceptibility-related genes must be identified to improve the detection of susceptibility or resistant individuals.

Additional studies are required to define in detail the mechanism of the association between susceptibility to leukemogenesis in response to BLV and polymorphism of MHC class II alleles. It has been postulated that the true disease-sensitivity gene is located near the MHC genes; therefore, MHC alleles may only be the marker genes (63). From the present study, however, it seems likely that quantitative and/or qualitative details of the immune reaction caused by particular allelic forms of MHC may contribute to the induction and maintenance of an efficient antitumor immune response and to the progression toward full-blown disease. By contrast, although the host’s genetic background, such as the HLA class II alleles, seems to be an important factor in determining whether carriers of HTLV-I develop either adult T-cell leukemia/lymphoma or HTLV-I-associated myelopathy, there seems to be no significant difference in frequencies of class II alleles between patients with adult T-cell leukemia/lymphoma and carriers of HTLV-I (64). Therefore, additional studies focusing on the amino acid residues that line the pocket that accommodates the side chain of a bound peptide may be useful in the case of HTLV-I. Aida et al. (12, 65) obtained evidence to suggest that a tumor-associated membrane glycoprotein that is serine-phosphorylated only during the leukemic stage of BLV-induced lymphoma is related to the BoLA-DR molecule. Therefore, alterations in the biochemical nature and physiological function of DR molecules in the progression of BLV-induced lymphoma, in association with resistance or susceptibility to tumor development, are also worthy of further examination.

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Yoshiko Nagaoka, Hidenori Kabeya, Misao Onuma, et al.


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