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Ovine MHC Class II *DRB1* Alleles Associated with Resistance or Susceptibility to Development of Bovine Leukemia Virus-induced Ovine Lymphoma[1]

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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 $\beta^{70/71}$ 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 $\beta^{70/71}$ 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 $\beta^{70/71}$, 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 immunoresponse 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

BLV[3] 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 it 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 tuberculoid 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-

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[3] The abbreviations used are: BLV, bovine leukemia virus; HTLV, human T-cell leukemia virus; PL, persistent lymphocytosis; MHC, major histocompatibility complex; HLA, human leukocyte antigen; BoLA, bovine lymphocyte antigen; OLA, ovine leukocyte antigen; Th, helper T cell; Tc, cytotoxic T cell; B, B cell; PBMC, peripheral blood mononuclear cells; PCR-RFLP, PCR-restriction fragment length polymorphism; RK, Arg-Lys; SR, Ser-Arg; QK, Gln-Lys; QT, Gln-Thr.

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 crossbreed) 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 (PQGRRRFGARAMVTYDCE; BLV Env 61–78); (*b*) Tc (GIFTLTWEIWGYDPLITFSL; BLV Env 121–140); and (*c*) B (PDCAICWEPSPPWAPE; BLV Env 177–192), which had been reported as Th, Tc, and B epitopes, respectively (41, 42). The peptides were mixed with complete Freund's adjuvant for the first and second immunizations and with incomplete Freund's adjuvant for the final immunization. The immunogens were injected i.m. every 2 weeks. One sheep was inoculated with PBS mixed with complete Freund's adjuvant and then with incomplete Freund's adjuvant as a control. Two weeks after the final immunization, all three of the sheep were challenged i.v. with 5×10^5 PBMC obtained from BLV-infected cattle.

Cells and Extraction of DNA. PBMC were separated from blood as described by Miyasaka and Trnka (43), and total chromosomal DNA was extracted from these cells as described by Hughes *et al.* (44). Tumor tissues were obtained from sheep with lymphoma and cut into slices about 0.5 cm in thickness and 1 cm^2 in area. They were frozen rapidly in liquid nitrogen and then stored at −80°C. Genomic DNA were prepared from these frozen blocks of tissue with 10% SDS and phenol-chloroform (45).

Amplification, Cloning, Screening, and DNA Sequencing of *OLA-DRB1*. Fifty ng of genomic DNA were subjected to amplification by PCR in a total volume of 50 μl of PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, and 120 μM dNTPs], to which oligonucleotide primers ERB3 and SRB3 (final concentration, 0.2 mM), and 2.5 units of rTaq polymerase (Toyobo Biochemicals, Tokyo, Japan) had been added. The locus-specific oligonucleotide primers ERB3, 5′-GGAATTCCTCTCTCTGCAGCACATTTCCT-3′, and SRB3, 5′-AAGTCGACCGCTGCACAGTGAAACTC-3′ were designed by reference to the intron-exon boundary regions of a *BoLA-DRB3* genomic clone (46). The underlined nucleotides are restriction sites for *Eco*RI and *Sal*I, respectively. The conditions for PCR were 94°C for 6 min, 61°C for 2 min, and 72°C for 2 min for the first cycle, which was followed by 35 cycles of 94°C for 1 min, 61°C for 2 min and 72°C for 2 min, and a final extension at 72°C for 10 min. All of the reactions were performed in a thermal cycler (PJ2000, Perkin-Elmer, Emeryville, CA).

The products of PCR were subcloned into the *Eco*RI and *Sal*I site of pBluescript II (SK^+; Stratagene, La Jolla, CA). The ligation mixture was used to transform *Escherichia coli* strain XL-I blue, and transformants were plated on agar plates with 100 μg/ml ampicillin, 40 μg/ml X-gal, and 20 μg/ml isopropylthio-β-D-galactoside, and incubated at 37°C overnight. Cells from white bacterial colonies were suspended in 30 μl (total volume) of PCR buffer, to which oligonucleotide primers (final concentration of 0.2 mM) T7, 5′-TAATACGACTCACTATAGGG-3′ and SB, 5′-GCCGCTCTAGAACTAGTGGATCCC-3′, and 2.5 units of rTaq 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 72°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 2% 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 *Rsa*I 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 dideoxy chain-termination method (47) with a *Bca*BEST 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 χ^2 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 *Sac*Ior *Hin*dIII and analyzed by Southern blotting (50) with, as a probe, the ^{32}P-labeled 6.9-kilobase *Eco*RI/*Sal*I 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 *Hin*dIII, 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 *Hin*dIII, which indicated that the PBMC from these animals consisted of polyclonal populations of various 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.

Table 1 *Characterization of sheep experimentally infected with BLV*

Sheep No.	Age		PBL[a]/mm^3	AtMC/mm^3	BLV provirus[b]		*OLA-DRB1* alleles[c] (A); (B)
	At infection	At death			*Hin*dIII	*Sac*I	
BLV-infected sheep with lymphoma							
pr 2274	0.5	7	3,501	22	2 bands	full	*OLA-DRB1*n19*; *OLA-DRB1*n23*
pr 2252	1	4	91,800	261,000	2 bands	full	*OLA-DRB1*n22*; *OLA-DRB1*n22*
pr 2212	1.5	3	15,724	13,728	2 bands	full	*OLA-DRB1*n1*; *OLA-DRB1*n2*
pr 2161	3	6	NT	NT	2 bands	full	*OLA-DRB1*n4*; *OLA-DRB1*n6*
pr 2044	1.5	5	1,209	145,886	2 bands	full	*OLA-DRB1*n3*; *OLA-DRB1*n10*
pr 2036	0.5	6	9,953	10,491	2 bands	full	*OLA-DRB1*n2*; *OLA-DRB1*n11*
pr 2012	0.5	5	9,202	430	2 bands	full	*OLA-DRB1*n26*; *OLA-DRB1*n2*
pr 2002	0.5	5	21,321	14,490	2 bands	full	*OLA-DRB1*n20*; *OLA-DRB1*n19*
pr 2001	5	10	1,058	164,934	2 bands	full	*OLA-DRB1*n4*; *OLA-DRB1*n23*
pr 1981	1	5	944	178,511	2 bands	full	*OLA-DRB1*n26*; *OLA-DRB1*n8*
pr 1979	0.5	5	3,175	146	2 bands	full	*OLA-DRB1*n26*; *OLA-DRB1*n1*
7389	1	6	7,600	NT	2 bands	full	*OLA-DRB1*n6*; *OLA-DRB1*n6*
7407	1	7	5,300	NT	2 bands	full	*OLA-DRB1*n20*; *OLA-DRB1*n10*
8554	0.5	6	682,670	NT	2 bands	full	*OLA-DRB1*n6*; *OLA-DRB1*n21*
pr 2208	0.5	6	10,290	58,423	2 bands	full	*OLA-DRB1*n2*; *OLA-DRB1*n2*
pr 2253	1	3	8,037	0	2 bands	full	*OLA-DRB1*n26*; *OLA-DRB1*n23*
BLV-infected but healthy sheep							
pr 2446	5	7	5,969	0	many	full	*OLA-DRB1*n7*; *OLA-DRB1*n23*
pr 2445	1	8	1,732	0	ND	ND (+)	*OLA-DRB1*n20*; *OLA-DRB1*n22*
pr 2423	3	9	NT	NT	ND	ND (+)	*OLA-DRB1*n9*; *OLA-DRB1*n2*
pr 2372	1	6	6,468	168	many	full	*OLA-DRB1*n9*; *OLA-DRB1*n20*
pr 2299	3	7	2,222	101	many	full	*OLA-DRB1*n25*; *OLA-DRB1*n23*
pr 2260	1	3	5,347	0	ND	ND (+)	*OLA-DRB1*n20*; *OLA-DRB1*n20*
pr 2259	1	3	3,874	31.5	many	full	*OLA-DRB1*n10*; *OLA-DRB1*n23*
pr 2258	1	3	4,704	64	many	full	*OLA-DRB1*n23*; *OLA-DRB1*n23*
pr 2257	1	3	3,822	137.5	many	full	*OLA-DRB1*n23*; *OLA-DRB1*n24*
pr 2256	1	3	3,515	79	many	full	*OLA-DRB1*n23*; *OLA-DRB1*n11*
pr 2254	1	3	5,054	76	many	full	*OLA-DRB1*n23*; *OLA-DRB1*n23*
pr 1978	0.5	5	4,628	468	many	full	*OLA-DRB1*n2*; *OLA-DRB1*n9*

[a] PBL, peripheral blood lymphocytes; AtMC, atypical mononuclear cells; NT, not tested; ND, not detected; (+), positive for the detection of BLV provirus integrated in chromosomal genome DNA by PCR with a BLV-specific primer.

[b] BLV provirus was detected by the Southern blotting technique using the ^{32}P-labeled 6.8-kb *Eco*RI/*Sal*I fragment of a BLV infectious molecular clone as a probe. When DNA was digested with *Sac*I, 6.8- and 1.3-kb fragments of integrated full-length BLV provirus were detected by hybridization. *Hin*dIII cuts once within the viral genome; "many" indicates that the BLV proviruses were integrated at many sites in the genomic DNA; "2 bands" indicates that the BLV provirus was integrated at one site.

[c] Both alleles, A and B, of each animal are shown.

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*n23* [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*n23* sequence that showed be 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*n23* allele was found most frequently in healthy sheep and appeared to encode amino acids RK at positions $\beta^{70/71}$. 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 $\beta^{70/71}$ 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 β^{70} as compared with 40.6% in the lymphoma group

Table 2 *OLA-DRB1 typing of sheep with BLV-induced lymphoma and healthy sheep*

Allele	Lymphoma ($n = 32$)		Healthy ($n = 24$)		P^a
	n	%	*n*	%	
*OLA-DRB1*n1*[b]	2[c]	6.3	0	0	0.5013
*OLA-DRB1*n2*[b]	5	15.6	2	8.3	0.6862
*OLA-DRB1*n3*[b]	1	3.1	0	0	>0.9999
*OLA-DRB1*n4*[b]	2	6.3	0	0	0.5013
*OLA-DRB1*n6*[b]	4	12.5	0	0	0.1268
*OLA-DRB1*n7*[b]	0	0	1	4.2	0.4286
*OLA-DRB1*n8*[b]	1	3.1	0	0	>0.9999
*OLA-DRB1*n9*[b]	0	0	3	12.5	0.0730
*OLA-DRB1*n10*[b]	2	6.3	1	4.2	>0.9999
*OLA-DRB1*n11*[b]	1	3.1	1	4.2	>0.9999
*OLA-DRB1*n19*	2	6.3	0	0	0.5013
*OLA-DRB1*n20*	2	6.3	4	16.7	0.3851
*OLA-DRB1*n21*	1	3.1	0	0	>0.9999
*OLA-DRB1*n22*	2	6.3	1	4.2	>0.9999
*OLA-DRB1*n23*	3	9.4	9	37.5	0.0195
*OLA-DRB1*n24*	0	0	1	4.2	0.4286
*OLA-DRB1*n25*	0	0	1	4.2	0.4286
*OLA-DRB1*n26*	4	12.5	0	0	0.1268

[a] Fisher's exact test was used to determine statistical significance.

[b] Alleles are different from the previously sequenced OLA-DRB1 alleles.

[c] Number of individuals positive for a particular DRB1 allele.

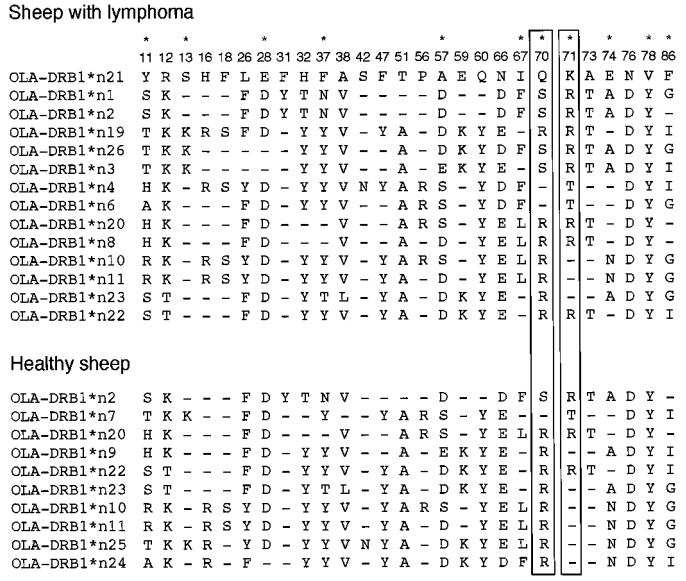

Fig. 1. Encoded amino acid residues that differ among *OLA-DRB1* alleles in BLV-infected sheep at the asymptomatic and lymphoma stages. Amino acid residues are indicated that are different among 18 distinct *OLA-DRB1* alleles. Numbers and ∗ *at the top*, the amino acid position numbers and putative antigen recognition sites; –, same as in the master sequence; *boxed*, residues at position 70 and 71.

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

	Lymphoma (n = 32)		Healthy (n = 24)		
Epitope	n	%	n	%	P^a
R^{70}	13[b]	40.6	21	87.5	0.0007
Non-R^{70}	19	59.4	3	12.5	0.0007
S^{70}	12	37.5	2	8.3	0.0146
Q^{70}	7	21.9	1	4.2	0.1196
K^{71}	7	21.9	16	66.7	0.0010
Non-K^{71}	25	78.1	8	33.3	0.0010
R^{71}	19	59.4	7	29.2	0.0324
T^{71}	6	21.4	1	4.2	0.2193
$R^{70}K^{71}$	6	18.8	16	66.7	0.0004
$R^{70}R^{71}$	7	37.5	5	20.8	>0.9999
$Q^{70}K^{71}$	1	3.1	0	0	>0.9999
Non-R^{70}, non-K^{71}	18	66.3	3	12.5	0.0009
$S^{70}R^{71}$	12	37.5	2	8.3	0.0146
$Q^{70}T^{71}$	6	18.8	1	4.2	0.2193

[a] Fisher's exact test was used to determine statistical significance.
[b] Number of alleles positive that encoded a particular epitope at position β^{70} and/or β^{71}.

(P = 0.0007). Moreover, 66.7% of healthy sheep carried alleles that encoded Lys at position β^{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*n9*, *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 β^{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 β^{70} as compared with 12.5% in the healthy group (P = 0.0007); 78.1% carried alleles encoding non-Lys amino acids at position β^{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 β^{70} and Arg at position β^{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.

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. Thus, resistance and 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. 2*B*). 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. 2*A*). 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 and then increased gradually throughout the experimental period. We next analyzed the proliferative response of PBMC

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

	Lymphoma (n = 16)		Healthy (n = 12)		
Genotype	n	%	n	%	P^a
RK/RK	0[b]	0	6	50.0	0.0025
SR/SR	4	25.0	0	0	0.1131
RK/SR	3	18.8	2	16.7	>0.9999
RK/QT	1	6.3	1	8.3	>0.9999
RK/RR	2	12.5	1	8.3	>0.9999
QK/QT	1	6.3	0	0	>0.9999
SR/RR	1	6.3	0	0	>0.9999
QT/QT	2	12.5	0	0	0.4921
RR/RR	2	12.5	2	16.7	>0.9999

[a] Fisher's exact test was used to determine statistical significance.
[b] Number of individual animals with a particular motif at positions $\beta^{70/71}$.

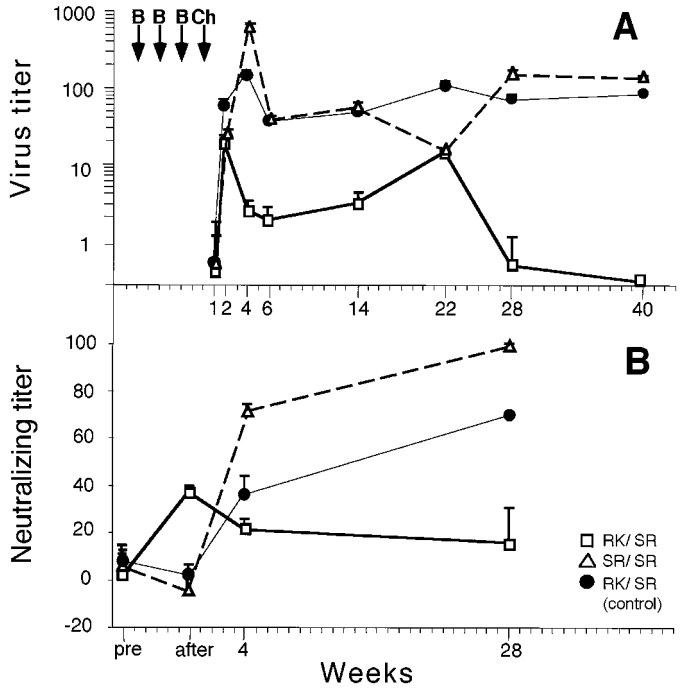

Fig. 2. Virus titer and neutralizing activities in BLV-challenged sheep. Sheep with either the *RK/SR* or the *SR/SR* genotype were immunized three times with a mixture of synthetic Th-, Tc-, and B-epitope peptides (*B-arrows*). Two weeks after the final immunization, all of the sheep were challenged with BLV (*Ch-arrow*). Sheep with the *RK/SR* genotype were inoculated with PBS instead of peptides as a control. *A*, the level of BLV in PBMC (virus titer) from BLV-challenged sheep was monitored by syncytium formation. PBMC were collected 1, 2, 4, 6, 14, 22, 28, and 40 weeks after the challenge with BLV. Triplicate cultures of PBMC ($5 \times l0^5$) 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. *B*, neutralizing activity in sera from BLV-challenged sheep, as assessed by inhibition of syncytium formation. Sera were prepared from blood before immunization (*pre*), 1 week after the final immunization (*after*), and 4 and 28 weeks after the BLV challenge. BLV-producing cell lines have been established from the fetal lamb kidney cells (FLK/BLV) (1×10^3) that had been productively infected with BLV and indicator cc8l cells (5×10^4) in 150 μl of RPMI 1640 supplemented with 10% heat-inactivated FCS were incubated with 50 μl of diluted serum (final dilution 1:10) for 24 h at 37°C in flat-bottomed 96-well microplates. The cells were then fixed as mentioned above. The percentage inhibition was calculated as:

$$1 - \frac{\text{Mean of the number of syncytia formed in a well to which serum had been added}}{\text{Mean number of syncytia in the control well}} \times 100$$

Bars, SD of mean for triplicated determinations.

to Th and Tc peptides and a mixture of the two peptides (Table 5). One week after the final immunization and 2 weeks after the BLV challenge, the level of incorporation of [^{3}H]thymidine by PBMC from 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,

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).

Test	Genotype		
	RK/SR	*SR/SR*	*RK/SR* (control)
Neutralizing activity after immunization with peptides	↑ [a]	→	→
Virus titer	↓	↑	↑
Lymphocyte proliferative response	↑	→	→
Progression of disease after challenge	Health	Lymphoma	Health

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

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 [^{3}H]thymidine was added to each well. Cells were harvested on glass-fiber filters, and the incorporation of [^{3}H]thymidine into DNA was determined with a liquid scintillation counter.

Genotype	Stimulation index (S.I.)[a] against peptide					
	1 week after the immunization			2 weeks after challenge with BLV		
	Th	Tc	Th + Tc	Th	Tc	Th + Tc
RK/SR	1.91 ± 0.50[b]	1.61 ± 0.07	2.16 ± 0.45	2.26 ± 0.06	1.27 ± 0.15	4.22 ± 1.52
SR/SR	1.09 ± 0.26	0.99 ± 0.11	1.22 ± 0.35	2.09 ± 0.10	0.6 ± 0.02	1.82 ± 0.16
RK/SR (control)	0.65 ± 0.13	0.67 ± 0.19	0.62 ± 0.06	1.23 ± 0.04	0.89 ± 0.13	1.12 ± 0.10

[a] S.I. = $\frac{\text{cpm in the stimulated culture}}{\text{cpm in the unstimulated control culture}}$.

[b] Mean ± SD of results of triplicate determinations.

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 $\beta^{70/71}$ 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 $\beta^{70/71}$? We respond: the amino acid residues 70–71 of the HLA-DRβ1 domain from peptide-binding pocket 4 (22). The amino acids at positions $\beta^{70/71}$ 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 disproportionate 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 $\beta^{70/71}$ 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 $\beta^{70/71}$ (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 $\beta^{70/71}$ 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 aetiologically 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 pathogenic 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 susceptibly 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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REFERENCES

1. Burny, A., Cleuter, Y., Kettmann, R., Mammerickx, M., Marbaix, G., Portetelle, D., van den Broeke, A., Willems, L., and Thomas, R. Bovine leukemia: facts and hypotheses derived from the study of an infectious cancer. Adv. Vet. Sci. Comp. Med., *32:* 149–170, 1988.
2. Coffin, J. M. Retroviridae: the viruses and their replication. *In:* B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus (eds.), Fields Virology, 2nd ed., Vol. 2, pp. 1767–1847. Philadelphia: Lippincott-Raven, 1996.
3. Sodroski, J. G., Rosen, C. A., and Haseltine, W. A. *trans*-acting transcriptional activation of the long terminal repeat of human T lymphotropic viruses in infected cells. Science (Washington DC), *225:* 381–385, 1984.
4. Sagata, N., Tsuzuku, K. J., Nagayoshi-Aida, M., Shimizu, F., Imagawa, K., and Ikawa, Y. Identification and some biochemical properties of the major X_{BL} gene product of bovine leukemia virus. Proc. Natl. Acad. Sci. USA, *82:* 7879–7883, 1985.
5. Hidaka, M., Inoue, J., Yoshida, M., and Seiki, M. Post-transcriptional regulator (rex) of HTLV-1 initiates expression of viral structural proteins but suppresses expression of regulatory proteins. EMBO J., *7:* 519–523, 1988.
6. Derse, D. *trans*-acting regulation of bovine leukemia virus mRNA processing. J. Virol., *62:* 1115–1119, 1988.
7. Djilali, S., Parodi, A-L., Levy, D., and Cockerell, G. L. Development of leukemia and lymphosarcoma induced by bovine leukemia virus in sheep: a hematopathological study. Leukemia (Baltimore), *1:* 777–781, 1987.

8. Aida, Y., Miyasaka, M., Okada, K., Onuma, M., Kogure, S., Suzuki, M., Minoprio, P., Levy, D., and Ikawa, Y. Further phenotypic characterization of target cells for bovine leukemia virus experimental infection in sheep. Am. J. Vet. Res., *50:* 1946–1951, 1989.
9. Murakami, K., Okada, K., Ikawa, Y., and Aida, Y. Bovine leukemia virus induces $CD5^-$ B cell lymphoma in sheep despite temporarily increasing $CD5^+$ B cells in asymptomatic stage. Virology, *202:* 458–465, 1994.
10. Murakami, K., Aida, Y., Kageyama, R., Numakunai, S., Ohshima, K., Okada, K., and Ikawa, Y. Immunopathological study and characterization of the phenotype of transformed cells in sheep with bovine leukemia virus-induced lymphosarcoma. Am. J. Vet. Res., *55:* 72–80, 1994.
11. Schwartz, I., and Lévy, D. Pathobiology of bovine leukemia virus. Vet. Res. (Paris), *25:* 521–536, 1994.
12. Aida, Y., Okada, K., and Amanuma, H. Phenotype and ontogeny of cells carrying a tumor-associated antigen that is expressed on bovine leukemia virus-induced lymphosarcoma. Cancer Res., *53:* 429–437, 1993.
13. Jorgensen, J. L., Reay, P. A., Ehrich, E. W., and Davis, M. M. Molecular components of T-cell recognition. Annu. Rev. Immunol., *10:* 835–873, 1992.
14. Germain, R. N., and Margulies, D. H. The biochemistry and cell biology of antigen processing and presentation. Annu. Rev. Immunol., *11:* 403–450, 1993.
15. Bjorkman, P. J., Saper, M. A., Samraoui, B., Bennett, W. S., Strominger, J. L., and Wiley, D. C. Structure of the human class I histocompatibility antigen, HLA-A2. Nature (Lond.), *329:* 506–512, 1987.
16. Brown, J. H., Jardetzky, T. S., Gorga, J. C., Stern, L. J., Urban, R. G., Strominger, J. L., and Wiley, D. C. Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. Nature (Lond.), *364:* 33–39, 1993.
17. Hammer, J., Takacs, B., and Sinigaglia, F. Identification of a motif for HLA-DR1 binding peptides using M13 display libraries. J. Exp. Med., *176:* 1007–1013, 1992.
18. Sette, A., Sidney, J., Oseroff, C., del Guercio, M. F., Southwood, S., Arrhenius, T., Powell, M. F., Colon, S. M., Gaeta, F. C., and Gray, H. M. HLA DR4w4-binding motifs illustrate the biochemical basis of degeneracy and specificity in peptide-DR interactions. J. Immunol., *151:* 3163–3170, 1993.
19. Falk, K., Rotzschke, O., Stevanovic, S., Jung, G., and Rammensee, H. G. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. Nature (Lond.), *351:* 290–296, 1991.
20. Rudensky, A. Y., Preston, H. P., Al-Ramadi, B. K., Rothbard, J., and Janeway, C. J. Truncation variants of peptides isolated from MHC class II molecules suggest sequence motifs. Nature (Lond.), *359:* 429–431, 1992.
21. Chicz, R. M., Urban, R. G., Lane, W. S., Gorga, J. C., Stern, L. J., Vignali, D. A., and Strominger, J. L. Predominant naturally processed peptides bound to HLA-DR1 are derived from MHC-related molecules and are heterogeneous in size. Nature (Lond.), *358:* 764–768, 1992.
22. Stern, L. J., Brown, J. H., Jardetzky, T. S., Gorga, J. C., Urban, R. G., Strominger, J. L., and Wiley, D. C. Crystal structure of the human class II MHC protein HLA-DR1 complexed with an influenza virus peptide. Nature (Lond.), *368:* 215–221, 1994.
23. Todd, J. A., Acha-Orbea,. H., Bell, J. I., Chao, N., Fronek, Z., Jacob, C. O., McDermott, M., Sinha, A. A., Timmerman, L., Steinman, L., and McDevitt, H. O. A molecular basis for MHC class II-associated autoimmunity. Science (Washington DC), *240:* 1003–1009, 1988.
24. Zerva, L., Cizman, B., Mehra, N. K., Alahari, S. K., Murali, R., Zmijewski, C. M., Kamoun, M., and Monos, D. S. Arginine at positions 13 or 70–71 in pocket 4 of *HLA-DRB1* alleles is associated with susceptibility to tuberculoid leprosy. J. Exp. Med., *183:* 829–836, 1996.
25. Hill, A. V., Allsopp, C. E., Kwiatkowski, D., Anstey, N. M., Twumasi, P., Rowe, P. A., Bennett, S., Brewster, D., McMichael, A. J., and Greenwood, B. M. Common West African HLA antigens are associated with protection from severe malaria. Nature (Lond.), *352:* 595–600, 1991.
26. Özdemir, E., Kakehi, Y., Nakamura, E., Kinoshita, H., Terachi, T., Okada, Y., and Yoshida, O. *HLA-DRB1*0101* and **0405* as protective alleles in Japanese patients with renal cell carcinoma. Cancer Res., *57:* 742–746, 1997.
27. Wank, R., and Thomssen, C. High risk of squamous cell carcinoma of the cervix for women with HLA- DQw3. Nature (Lond.), *352:* 723–725, 1991.
28. Glew, S. S., Stern, P. L., Davidson, J. A., and Dyer, P. A. HLA antigens and cervical carcinoma. Nature (Lond.), *356:* 22, 1992.
29. Lee, J. E., Reveille, J. D., Ross, M. I., and Platsoucas, C. D. *HLA-DQB1*0301* association with increased cutaneous melanoma risk. Int. J. Cancer, *59:* 510–513, 1994.
30. Lewin, H. A., and Bernoco, D. Evidence for BoLA-linked resistance and susceptibility to subclinical progression of bovine leukemia virus infection. Anim. Genet., *17:* 197–207, 1986.
31. Lewin, H. A., Wu, M-C., Stewart, J. A., and Nolan, T. J. Association between *BoLA* and subclinical bovine leukemia virus infection in a herd of Holstein-Friesian cows. Immunogenetics, *27:* 338–344, 1988.
32. Stear, M. J., Dimmock, C. K., Newman, M. J., and Nicholas, F. W. BoLA antigens are associated with increased frequency of persistent lymphocytosis in bovine leukemia virus infected cattle and with increased incidence of antibodies to bovine leukemia virus. Anim. Genet., *19:* 151–158, 1988.
33. van Eijk, M. J., Stewart, H. J. A., Beever, J. E., Fernando, R. L., and Lewin, H. A. Development of persistent lymphocytosis in cattle is closely associated with DRB2. Immunogenetics, *37:* 64–68, 1992.
34. Xu, A., van Eijk, M. J., Park, C., and Lewin, H. A. Polymorphism in *BoLA-DRB3* exon 2 correlates with resistance to persistent lymphocytosis caused by bovine leukemia virus. J. Immunol., *151:* 6977–6985, 1993.
35. Zanotti, M., Poli, G., Ponti, W., Polli, M., Rocchi, M., Bolzani, E., Longeri, M., Russo, S., Lewin, H. A., and van Eijk, M. J. T. Association of BoLA class II haplotypes with subclinical progression of bovine leukemia virus infection in Holstein-Friesian cattle. Anim. Genet., *27:* 337–341, 1996.
36. Aida, Y., Kohda, C., Morooka, A., Nakai, Y., Ogimoto, K., Urao, T., and Asahina, M. Cloning of cDNAs and the molecular evolution of a bovine MHC class II *DRA* gene. Biochem. Biophys. Res. Commun., *204:* 195–202, 1994.
37. Nishino, Y., Tajima, S., and Aida, Y. Cattle cDNA clone encoding a new allele of the MHC class II *DQA1* gene. Immunogenetics, *42:* 306–307, 1995.
38. Morooka, A., Asahina, M., Kohda, C., Tajima, S., Niimi, M., Nishino, Y., Sugiyama, M., and Aida, Y. Nucleotide sequence and the molecular evolution of a new *A2* gene in the *DQ* subregion of the bovine major histocompatibility complex. Biochem. Biophys. Res. Commun., *212:* 110–117, 1995.
39. Aida, Y., Niimi, M., Asahina, M., Okada, K., Nakai, Y., and Ogimoto, K. Identification of a new bovine MHC class II *DRB* allele by nucleotide sequencing and an analysis of phylogenetic relationships. Biochem. Biophys. Res. Commun., *209:* 981–988, 1995.
40. Dikiniene, N., and Aida, Y. Cattle cDNA clones encoding MHC class II *DQB1* and *DQB2* genes. Immunogenetics, *42:* 75, 1995.
41. Gatei, M. H., Naif, H. M., Kumar, S., Boyle, D. B., Daniel, R. C., Good, M. F., and Lavin, M. F. Protection of sheep against bovine leukemia virus (BLV) infection by vaccination with recombinant vaccinia viruses expressing BLV envelope glycoproteins: correlation of protection with CD4 T-cell response to gp51 peptide 51–70. J. Virol., *67:* 1803–1810, 1993.
42. Callebaut, I., Voneche, V., Mager, A., Fumiere, O., Krchnak, V., Merza, M., Zavada, J., Mammerickx, M., Burny, A., and Portetelle, D. Mapping of B-neutralizing and T-helper cell epitopes on the bovine leukemia virus external glycoprotein gp51. J. Virol., *67:* 5321–5327, 1993.
43. Miyasaka, M., and Trnka, Z. Sheep as an experimental model for immunology: immunological techniques *in vitro* and *in vivo. In:* I. Lefkovits and B. Pernis (eds.), Immunological Methods, Vol. 3, pp. 403–422. New York: Academic Press, Inc., 1985.
44. Hughes, S. H., Shank, P. R., Spector, D. H., Kung, H-J., Bishop, J. M., Varmus, H. E., Vogt, P. K., and Breitman, M. L. Proviruses of avian sarcoma virus are terminally redundant, co-extensive with unintegrated linear DNA and integrated at many sites. Cell, *15:* 1397–1410, 1978.
45. McKnight, G. S. The induction of ovalbumin and conalbumin mRNA by estrogen and progesterone in chick oviduct explant cultures. Cell, *14:* 403–413, 1978.
46. van Eijk, M. J., Stewart-Haynes, J. A., and Lewin, H. A. Extensive polymorphism of the *BoLA-DRB3* gene distinguished by PCR-RFLP. Anim. Genet., *23:* 483–496, 1992.
47. Sanger, F., Nicklen, S., and Coulson, A. R. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA, *74:* 5463–5467, 1977.
48. Devereux, J., Haeberli, P., and Smithies, O. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res., *12:* 387–395, 1984.
49. Kabeya, H., Ohashi, K., Ohishi, K., Sugimoto, C., Amanuma, H., and Onuma, M. An effective peptide vaccine to eliminate bovine leukemia virus (BLV) infected cells in carrier sheep, Vaccine, *14:* 1118–1122, 1996.
50. Sambrook, J., Fritsch, E. F., and Maniatis, T. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1989.
51. Inabe, K., Ikuta, K., and Aida, Y. Transmission and propagation in cell culture of virus produced by cells transfected with an infectious molecular clone of bovine leukemia virus. Virology, *245:* 53–64, 1998.
52. Tajima, S., Ikawa, Y., and Aida, Y. Complete bovine leukemia virus (BLV) provirus is conserved in BLV-infected cattle throughout the course of B-cell lymphosarcoma development. J. Virol., *72:* 7569–7576, 1998.
53. Schwaiger, F. W., Weyers, E., Buitkamp, J., Ede, A. J., Crawford, A., and Epplen, J. T. Interdependent *MHC-DRB* exon-plus-intron evolution in artiodactyls. Mol. Biol. Evol., *11:* 239–249, 1994.
54. Ballingall, K. T., Wright, H., Redmond, J., Dutia, B. M., Hopkins, J., Lang, J., Deverson, E. V., Howard, J. C., Puri, N., and Haig, D. Expression and characterization of ovine major histocompatibility complex class II (*OLA-DR)* genes. Anim. Genet., *23:* 347–359, 1992.
55. Ballingall, K. T., Dutia, B. M., Hopkins, J., and Wright, H. Analysis of the fine specificities of sheep major histocompatibility complex class II-specific monoclonal antibodies using mouse L-cell transfectants. Anim. Genet., *26:* 79–84, 1995.
56. Fu, X. T., Bono, C. P., Woulfe, S. L., Swearingen, C., Summers, N. L., Sinigaglia, F., Sette, A., Schwartz, B. D., and Karr, R. W. Pocket 4 of the HLA-DR(α,β 1*0401) molecule is a major determinant of T cell recognition of peptide. J. Exp. Med., *181:* 915–926, 1995.
57. Richeldi, L., Sorrentino, R., and Saltini, C. *HLA-DPB1* glutamate 69: a genetic marker of beryllium disease. Science (Washington DC), *262:* 242–244, 1993.
58. Hammer, J., Gallazzi, F., Bono, E., Karr, R. W., Guenot, J., Valsasnini, P., Nagy, Z. A., and Sinigaglia, F. Peptide binding specificity of HLA-DR4 molecules: correlation with rheumatoid arthritis association. J. Exp. Med., *181:* 1847–1855, 1995.
59. Wank, R., Schendel, D. J., and Thomssen, C. HLA antigens and cervical carcinoma. Nature (Lond.), *356:* 22–23, 1992.
60. Han, R., Breitburd, F., Marche, P. N., and Orth, G. Linkage of regression and malignant conversion of rabbit viral papillomas to MHC class II genes. Nature (Lond.), *356:* 66–68, 1992.
61. zur Hausen, H. Viruses in human cancers. Science (Washington DC), *254:* 1167–1173, 1991.
62. Tajima, S., Zhuang, W. Z., Kato, M. V., Okada, K., Ikawa, Y., and Aida, Y. Function and conformation of wild-type p53 protein are influenced by mutations in bovine leukemia virus-induced B-cell lymphosarcoma. Virology, *243:* 235–246, 1998.
63. Rukstalis, D. B., Bubley, G. J., Donahue, J. P., Richie, J. P., Seidman, J. G., and DeWolf, W. C. Regional loss of chromosome 6 in two urological malignancies. Cancer Res., *49:* 5087–5090, 1989.
64. Manns, A., Hanchard, B., Morgan, O. S., Wilks, R., Cranston, B., Nam, J. M., Blank, M., Kuwayama, M., Yashiki, S., Fujiyoshi, T., Blattner, W., and Sonoda, S. Human leukocyte antigen class II alleles associated with human T-cell lymphotropic virus type I infection and adult T-cell leukemia/lymphoma in a Black population. J. Natl. Cancer Inst., *90:* 617–622, 1998.
65. Aida, Y., Okada, K., Ohtsuka, M., and Amanuma, H. Tumor-associated M_r 34,000 and M_r 32,000 membrane glycoproteins that are serine phosphorylated specifically in bovine leukemia virus-induced lymphosarcoma cells. Cancer Res., *52:* 6463–6470, 1992.