

GENETIC ASPECTS OF CANINE MIXED LEUKOCYTE CULTURES*

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(Received for publication 10 June 1974)

One genetic region (the major histocompatibility complex [MHC]¹) is of major importance for the prognosis of graft survival. This region has been identified in all mammalian species so far investigated and is localized on an autosomal chromosome. It has in man been called HL-A, in mouse H-2, and in dogs DL-A.

The observation that the lymphocytes of the great majority of HL-A identical siblings, i.e. identical for the serologically defined (SD) histocompatibility antigens, do not stimulate each other in the mixed leukocyte culture (MLC) test, initially led to the assumption that proliferation of lymphocytes in this test might be brought about by SD differences. However, in a small number of families MLC stimulation was found between SD identical siblings (references 1-5 and footnote 2). This observation led to the postulation of a genetic region on the same chromosome as, but distinct from the SD loci, controlling structures determining reactivity in the MLC test. These latter structures have been called lymphocyte-defined (LD) determinants. The stimulation found in MLC tests between SD identical siblings can thus be explained by a recombination between the SD and LD region of the MHC in man. The frequency of such recombinations in man is calculated to be approximately 1%.² The system controlling LD determinants shows a high degree of polymorphism since negative MLC reactions are almost never found between unrelated SD nonidentical individuals nor among pairs of individuals chosen at random. However, a few well documented cases of nonstimulation in MLC between SD nonidentical unrelated individuals are now available (references 6-8). When MLC tests are performed between SD identical unrelated individuals, about 10% of the reactions are found to

* Supported in part by the National Institute of Health (contract PH43-65-992), the J.A. Cohen Institute for Radiopathology and Radiation Protection (IRS), The Dutch Organization for Medical Research (FUNGO) which is subsidized by the Dutch Organization for the Advancement of Pure Research (ZWO), The Dutch Organization for Health Research (TNO), The Dutch Kidney Foundation and the Whitehall Foundation.

† Faculty Research Awardee of the American Cancer Society.

¹ *Abbreviations used in this paper:* LD, lymphocyte defined; MHC, major histocompatibility complex; MLC, mixed leukocyte culture; SD, serologically defined.

² Keuning, J. J., J. G. van den Tweel, A. Termijtelen, and J. J. van Rood. 1974. An estimation of the recombination fraction between the MLC locus and the Four locus. Manuscript for publication.

be negative (9–11, and our own unpublished observations). This suggests a positive association or linkage disequilibrium between some LD and SD determinants.

In the dog, as in man, mouse, and Rhesus monkey, two closely linked loci have been defined, alleles of which determine the DL-A SD antigens (12). The antigens have thus been separated into the first and second segregant series of antigens. We will refer to these loci as SD-1 and SD-2 respectively. Fig. 1 presents a schematic view of the MHC in the dog and the antigens coded for by the SD-1 and SD-2 loci.

The analysis of the genetic control of the MLC reaction in dogs has only recently been initiated. It has been demonstrated that no significant MLC stimulation occurs between SD identical dog siblings (13). Data have been obtained suggesting that MLC reactivity is controlled by genes on one chromosomal locus, localized outside the second series locus (14). Recent data in different mammalian species (15–19) stress the importance of matching donors and recipients of organ transplantations for LD as well as for SD determinants. For a thorough experimental evaluation of the influence of LD and SD structures on graft survival a model in a noninbred experimental animal is necessary. The present studies were initiated in dogs to create such a model. In this paper it will be demonstrated that in the dog there exists at least one LD locus which is polymorphic and distinct from, but closely linked to, the already known SD loci (12). There appears to be a pronounced linkage disequilibrium between canine SD and LD alleles.

Materials and Methods

Dogs.—

Unrelated animals: 180 dogs were investigated at the first international dog workshop on Canine Immunogenetics in Rotterdam, The Netherlands in 1972 (14). From this material 45 unrelated animals with 18 SD phenotypes were selected. The maximum number of animals in a group of phenotypically SD identical unrelated dogs was four. 36 animals were mongrel dogs, 2 were purebred labrador retrievers, and 7 were purebred beagles. The dogs of the two latter groups all belonged to different SD phenotypes.

Families: In additional DL-A identical siblings were selected from an outbred beagle colony of the Centraal Proefdieren Bedrijf TNO, Austerlitz, The Netherlands, (39 in total) and from the mongrel dog families studied during the workshop (13 in total). Two complete families were tested. One dog in each family carried a recombinant chromosome with a recombination between SD-1 and SD-2 of DL-A; in one family a third dog carried a presumed recombination between LD and SD-1.

Serology.—DL-A typing was performed using a one stage microcytotoxicity test as described elsewhere (20). A battery of 80 cytotoxic sera were used to define the following DL-A antigens: DL 1, 2, 3, 7, 8, 9, 10 of the first series, and DL 4, 5-13, 6, 11, 12, 13-5, 14 of the second series. (see Fig. 1) The DL-14 sera were kindly provided by Doctors E. D. Thomas and F. R. Storb. Both series still have unidentified or "blank" alleles (14).

MLC Test.—MLC tests were performed using the Hartzman Bach micromethod (21) modified for the dog (M. L. Bach, unpublished data). Blood was drawn under sterile conditions from the jugular vein or vena cephalica anterior by puncture and collected in bottles with thromboliquine (Organon, Inc., Oss, The Netherlands), 100 U/ml blood. Whole blood was centrifuged at 250 g for 10 min in 50 ml Falcon tubes (catalogue no. 2070, Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). The obtained buffy coat was removed and centrifuged again for 10 min at 180 g. Supernatant plasma was pooled, heat inactivated, and centrifuged at 900 g

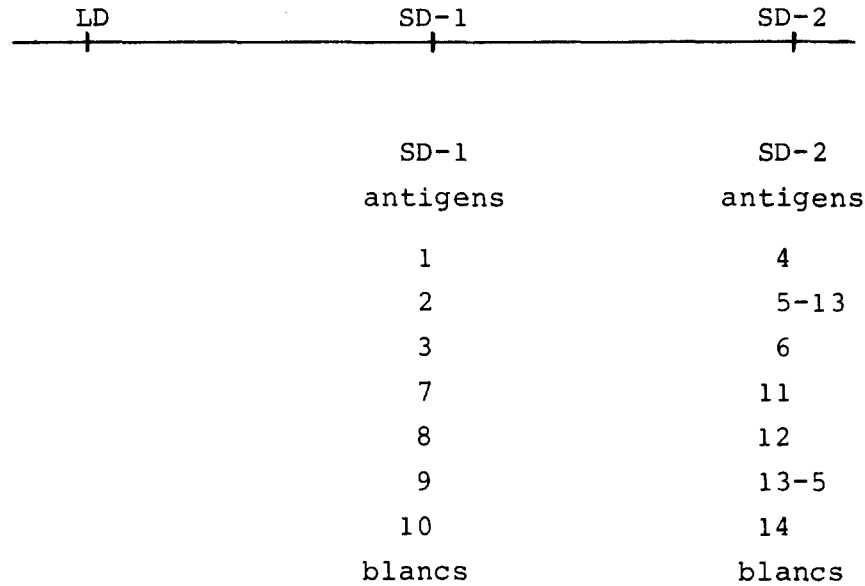


FIG. 1. The MHC in the dog.

for 10 min. A total vol of cells and plasma of 15 ml was resuspended and under-layered with 13–14 ml of lymphoprep (Nyegaard and Co., Oslo, Norway) and centrifuged for 15 min at 400 *g*. The lymphocyte-rich interface was harvested and washed twice in RPMI 1640 supplemented with 100 U penicillin/100 μ g streptomycin/ml and 0.3% thrombolyquine.

An aliquot of this cell suspension (to be used as stimulator cells) was incubated with 25 μ g mitomycin C (A. Christiaens S. A. Brussels, Belgium) for 30 min, washed twice, and collected at 180 *g*. Responder cells were prepared in suspensions of 1×10^6 cells/ml culture medium. Stimulator cells were suspended at a concentration of 4×10^6 cells/ml tissue culture medium (RPMI 1640, 100 U penicillin/100 μ g streptomycin, 0.06 mmol glutamine [all from Flow Laboratories, Amersham, Scotland], 0.3% thrombolyquine, and 15% heat-inactivated pooled dog plasma).

0.1-ml aliquots of responder and 0.1-ml aliquots of stimulator cells were mixed and incubated in wells in a Linbro plate (catalogue no. IS-FB-96-TC, Linbro Chemical Co., New Haven, Conn.) for 7 days in a humidified CO₂ incubator at 37°C. All assays were performed in triplicate. At day 6, 2 μ Ci [³H]thymidine (sp act 2 Ci/mmol, The Radiochemical Centre, Amersham, England) was added to each culture. The cells were harvested 18 h later on millipore glassfiber filters with an automatic sample harvester (22) using saline for washing. Each cell combination was assayed in triplicate and each experiment was repeated once or twice. Log converted counts of each combination (XYm) are compared with the autologous value (XXm). The comparison is made by means of a *t* test. A combination is considered to be negative when the (one sided) *t* test is not significant at the 5% level (critical *t* value is 2.13, one sided, 4 degrees of freedom, $\alpha = 0.05$).

RESULTS

SD identical unrelated dogs were divided into two groups. The first consisted of 13 mongrel dogs, having four DL antigens in common (so-called "full house identicals"). Animals which had only three DL antigens in common and one

antigen unknown, were put in a second group. The possibility that some of these latter animals differ for a DL antigen which was not recognized by the available antisera could not be excluded. The possibility that homozygosity for one DL group existed in any of these latter animals could also not be excluded. 9 out of the total number of 32 dogs in this group were beagles or labrador retrievers. However, they belonged to different SD phenotypes and were not tested in between each other.

In Table I the results of the MLC tests performed between these SD identical unrelated dogs are given. In the group of full house identical dogs the percentage of negative combinations in the MLC test was 71 by the criteria stated. In the

TABLE I
Results of Unilateral MLC Tests in Unrelated Dogs

No. of DL groups in common	No. of different SD phenotypes	No. of dogs	No. of negative MLC tests per total number of MLC tests performed
4	6	13	10/14, 71%
Full house identicals			$P < 0.01$
3	12	32	21/62, 33%
Identical for first locus antigens	7	19	17/38, 47%
Identical for second locus antigens	5	13	4/24, 17%
			$P < 0.02$
0			
Full house nonidentical	8	16	0/36, 0%

group of dogs with one DL antigen missing, the percentage of negative combinations was 33. When this group, however, was split up into a group with the two first series antigens known, and a group in which the two second series antigens could be determined, it appeared that identity for first series antigens resulted in 47% negative MLC tests, whereas identity for the second series gave only 17% negative combinations. The association of the LD locus with the two SD loci was further evaluated by selecting one dog from each negative pair and testing these animals in between each other. The dogs were investigated in a combinatorial MLC test (Table II). In this table only the 10 relevant dogs are represented. In this experiment 13 of the 210 (6.2%) one-way MLC combinations were negative according to our criteria. This experiment was performed twice. The cpm in Table II are those of the first experiment; 12 discrepancies between results of the first and the second experiment are represented by the highest values. The reproducibility of the negative combinations in the first and second experiment was excellent. Table III presents the results of the MLC test performed between DL-A identical dog siblings. No positive tests were found in the 98 unilateral combinations studied.

TABLE II
MLC test between 10 unrelated dogs selected from identical DL-A phenotypes

Dog no.	DL-A type	02m*	073m	1358m	1373m	011m	1338m	03m	072m	C94m	024m
02	3, 9, 4	356(42)‡	11,331(12)	2,709(54)§	63,028(22)	11,046(66)	8,096(82)	60,975(29)	7,660(32)	561(3)§	41,477(65)
073	3, 9, 4	6,829(86)	778(62)	1,776(91)§	88,498(15)	35,491(31)	39,729(44)	69,507(4)	14,867(60)	1,231(9)	41,200(38)
1358	3, 7, 4, 11	1,314(98)§	292(14)§	432(3)	2,722(53)	53,651(5)	8,902(49)	58,595(26)	2,468(98)§	6,770(46)	83,146(45)
1373	3, 7, 4, 11	34,356(10)	34,557(27)	3,887(80)§	1,666(42)	52,268(36)	15,896(76)	47,001(24)	29,251(77)	20,872(47)	42,844(73)
011	3, 7, 5+13	28,994(9)	30,965(26)	12,435(63)	36,199(32)	146(34)	12,450(89)	42,716(6)	8,469(49)	64,793(20)	7,127(72)
1338	3, 9, 5+13	32,798(13)	22,604(47)	1,628(73)§	28,878(62)	1,974(39)§	599(37)	77,825(4)	12,909(25)	2,816(99)§	1,902(38)
03	9, 4, 5+13	41,863(46)	30,158(54)	26,980(81)	67,297(27)	4,816(85)	40,438(21)	370(17)	8,837(73)	20,811(49)	7,451(80)
072	9, 4, 5+13	44,496(3)	38,689(11)	51,403(2)	32,922(5)	35,463(8)	34,682(18)	24,757(11)	1,054(23)	44,433(24)	44,944(12)
C94	3, 11, 12	666(35)§	642(26)§	9,561(81)	8,322(30)	56,855(13)	4,075(50)	72,570(20)	5,787(39)	532(29)	75,766(12)
024	3, 11, 5+13	93,542(36)	65,402(4)	24,074(65)	90,871(3)	4,072(96)§	11,242(74)	68,938(28)	7,301(97)	101,951(21)	850(44)

* m, mitomycin treated.
‡ cpm, in parenthesis: coefficient of variation.
§ Negative values.

A family wherein a crossover occurred between the first and the second segregant series of DL-A is illustrated in Table IV. Pup 659 carries the presumed recombinant chromosome. Since one of the maternal chromosomes in which the crossover event occurred carries a "blank" allele at SD-2 it is more difficult to be certain of this recombinatorial event than if the mother had had all four antigens identified (see Fig. 2). Hypothetical alleles A, B, C, and D are assigned at LD. Siblings 658 and 659 are, as a result of this presumed recombinatorial event,

TABLE III
Results of Unilateral MLC Tests in SD Identical Dog Siblings

Identified DL antigens in common	No. of different SD phenotypes	No. of dogs	No. of negative MLC tests/total number of MLC tests performed
4	6	17	34/34
3	11	31	60/60
2	2	4	4/4
Total	19	52	98/98

TABLE IV
Unilateral MLC Tests in a Family with a Recombinant between First and Second Segregant Series of DL-A

Dog	DL-A genotype	153m*	657m	658m	659m	660m
Mother (deceased)	7, 11/3-					
Father	153 1, 13/9, 4	230(40)‡	23, 516(24)	20, 934(2)	88, 081(17)	17, 209(39)
Sibling	657 1, 13/3-	85, 091(7)	163(18)	44, 853(43)	82, 591(12)	340(42)
Sibling	658 9, 4/3-	77, 944(4)	63, 712(10)	389(43)	68, 464(2)	65, 010(15)
Sibling (recombinant)	659 9, 4/7-	92, 844(17)	74, 557(17)	45, 337(16)	135(28)	84, 720(1)
Sibling	660 1, 13/3-	96, 422(7)	151(14)	31, 659(29)	76, 301(11)	336(35)

* m, mitomycin treated.

‡ cpm, in brackets coefficient of variation.

identical for both second series antigens (one of which is unidentified by the available antisera as discussed above). The MLC test between them, however, is positive in both directions indicating disparity at LD as illustrated in Fig. 2. The unilateral MLC tests between the DL-A identical siblings 657 and 660 are both negative, indicating LD identity as well as SD identity.

Results obtained in a second family are shown in Table V. The parents are unrelated, they share the haplotype 10, 5-13 (see Fig. 3). Hypothetical alleles W, X, X', and Y are assigned at LD. Also in this family there is a child bearing a recombinant chromosome originating from the paternal haplotypes, the crossover occurred between SD-1 and SD-2. This dog 016 stimulates and is stimulated by all other family members tested. This finding is expected since as a

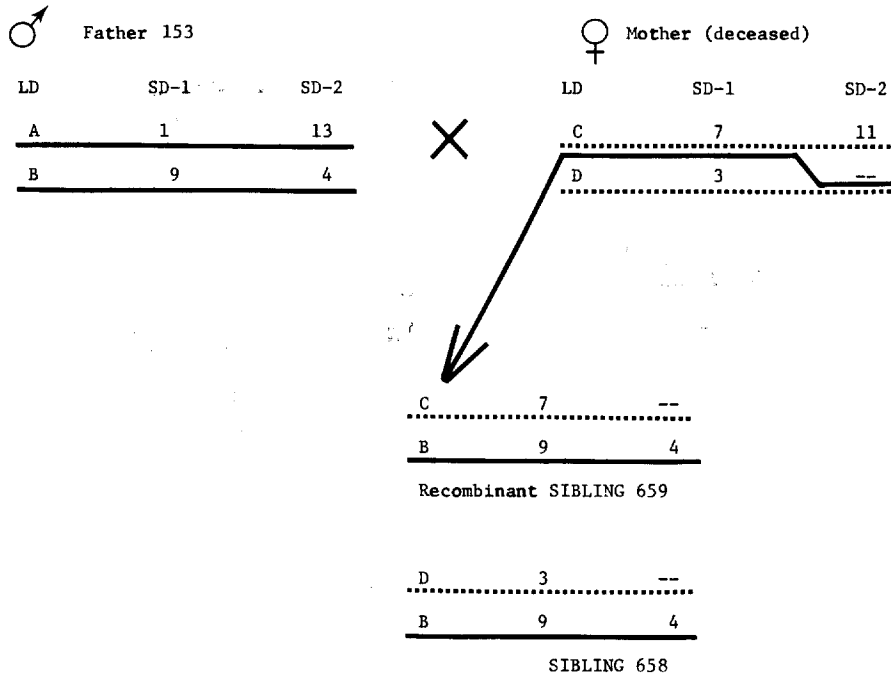


FIG. 2. Family table IV.

TABLE V

Unilateral MLC Test in a Family with a Recombination between SD-1 and SD-2 (DL-A) and a Recombination between SD-1 and the LD Locus

Dog	DL-A genotype	1293m*	602m	015m	016m	017m	018m
Mother	1293 3, -/10, 5-13	144 (22)‡	28,607 (15)	249 (45)	38,312 (66)	249 (28)	817 (88)
Father	602 7, 12/10, 5-13	18,521 (10)	151 (4)	29,844 (50)	51,312 (2)	542 (21)	52,581 (5)
Sibling	015 3, -/10, 5-13	186 (2)	30,367 (19)	174 (17)	56,843 (10)	374 (55)	347 (55)
Sibling (SD re-combinant)	016 3, -/7, 5-13	8,420 (49)	39,795 (14)	27,778 (11)	176 (27)	55,405 (20)	56,691 (7)
Sibling (LD re-combinant)	017 7, 12/10, 5-13	7,542 (7)	48,085 (18)	50,599 (19)	57,329 (10)	142 (13)	52,328 (16)
Sibling	018 3, -/10, 5-13	142 (7)	49,021 (14)	204 (41)	71,508 (8)	351 (5)	186 (16)

* m, mitomycin treated.

‡ cpm, in brackets coefficient of variation.

result of the crossover event sibling 016 becomes W, Y at the LD locus, differing from all other family members. The unilateral MLC tests performed between the mother (1293) and her SD identical pups (015) and (018) are respectively negative and very low. The father is very weakly (according to the *t* test) stimulated by the cells of his SD identical child 017; the reverse action, however, is

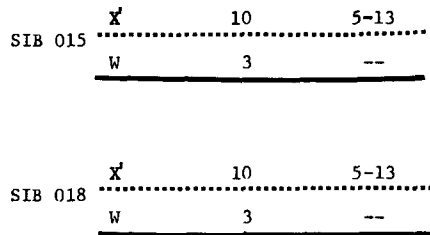
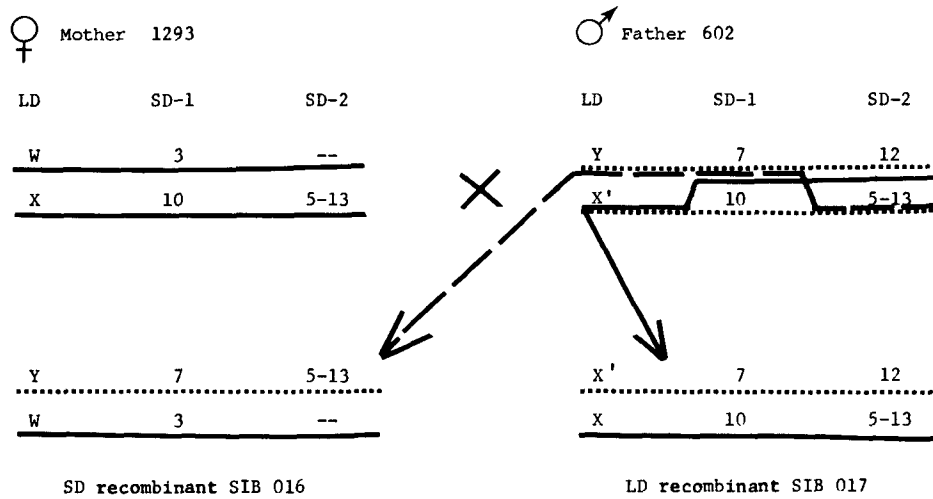


FIG. 3. Family table V.

strongly positive. Furthermore, it can be observed that pup 017 only stimulates recombinant 016, but that 017 is stimulated by all family members tested; this is consistent with homozygosity at LD in sibling 017 as diagramed in Fig. 3, which implies a recombination between the SD-1 and LD loci. In further studies the cells of this animal proved to be useful in the LD typing of unrelated animals (unpublished observations).

DISCUSSION

In three mammalian species, man (1-5), mouse (23), and Rhesus monkey (24), evidence has been obtained for the existence of a genetic system which controls reactivity in MLC (the LD locus) and which is linked to, but distinct from the loci coding for the SD major histocompatibility antigens. The present investigation addresses itself to the exploration of a LD locus in dogs. The

results obtained indicate that an LD locus separate from the loci coding for the SD antigens must exist in the dog. This is based on the finding of a presumed crossover between the LD and SD region in the family shown in Table V and on the finding of negative MLC tests in SD nonidentical dogs. Both phenomena will be discussed below.

The results of MLC tests in SD identical unrelated dogs as shown in Table I show a high percentage of negative combinations in the "full house" identical group (71%). This might be due to a strong linkage disequilibrium between SD and LD loci or to a low polymorphism of the LD system. The low percentage (6.2%) found of LD identical SD nonidentical individuals, thus points to a strong linkage disequilibrium as the explanation. The lower percentage (33%) of negative MLC tests, in the nonfull house SD identical group might be due to incomplete SD typing. SD differences may be present in animals which are thought to be SD identical. The observation that dogs which are identical for the first series antigens appear to be MLC nonstimulatory more often (47%) than dogs identical for the second series antigens (17%) suggests that the LD locus in the dog is associated with SD-1 rather than SD-2.

These observations are supported by the finding that dogs 658 and 659 in Table IV do stimulate each other in the MLC test. If the LD locus was located near or outside SD-2, it could be expected that both animals would be LD identical contrary to the results obtained. The same holds for 016 (the SD recombinant) and for the siblings 015 and 018 shown in Table V. An LD locus located outside the second series locus should result in a negative MLC test between these dogs, again in contrast to the results obtained.

The localization of the LD locus within the MHC in dogs is found in the family of Table V. Sibling 017 is stimulated by all other family members, but, with the exception of 016, does not stimulate any of them. This indicates that 017 is homozygous for the LD antigens as schematically proposed in Fig. 3. The homozygosity of 017 is most likely the result of a recombination between the LD and the SD region of the chromosome. Offspring of this dog will be raised to provide the ultimate proof of this recombinatorial event.

A double recombination is a less likely event than the proposed single recombination between LD and SD-1. The reciprocal stimulation between sibling 016 and siblings 015 and 018 excludes localization of the LD locus outside the second series locus. The LD locus in the dog is therefore in all probability localized outside SD-1 (Fig. 1) in contrast to earlier reported findings (14).

The linkage disequilibrium found between SD and LD loci in dogs is more pronounced than that observed in man. One of the possible explanations might be a founder or bottleneck effect in which special gene combinations are overrepresented in subsequent generations due to a limited number of mating animals in some stage(s) of dog history (25).

Another explanation of the observed high linkage disequilibrium in the dog might be due to a relatively recent mixing of inbred populations of animals, so

that the alleles found in the different inbred populations have not yet had time to reach linkage equilibrium in the noninbred population (26). This situation in dogs might be analogous to that observed in plants where a high level of linkage disequilibrium is found for isozyme markers where local inbred populations have been recently mixed (27).

Epstein et al. (28) and Westbroek et al. (29) have documented that SD typing and matching with a limited number of unrefined antisera improved canine bone marrow and small intestine graft survival. This is in strong contrast with the experience in human and Rhesus monkey allografts where such a beneficial effect was not found. In retrospect the explanation for this discrepancy between primates and dogs might be the high linkage disequilibrium between LD and SD in the latter species. SD matching there will often include an unintentional LD typing.

In the combinatorial MLC test (Table II) 13 of the 210 combinations are repeatedly negative. As these dogs are not identical for the SD system, this is another proof for a LD locus distinct from the known SD loci. The selection procedure used makes it likely that we are dealing with LD determinants that occur rather frequently.

On the assumption that all MLC determinants have the same strength and frequency it can be calculated that the number of alleles at the LD locus is about seven (30). This is in accordance with the findings of Grosse-Wilde et al. (31) who found with the help of LD homozygous cells that there are at least seven different LD alleles. The number of LD alleles calculated from our studies is probably too low since the assumption of equal strength and frequency of MLC determinants is not correct. Another reason for underestimating the number of LD alleles is the fact that nearly all dogs tested have a SD-1 antigen in common. The high linkage disequilibrium between SD-1 and LD will have limited the variability of the LD system in this group of dogs.

The existence of a separate MLC locus was not apparent from the dog siblings studied. No recombination between SD and LD loci could be demonstrated. Although the exact recombination frequency between SD and LD loci in dogs still has to be determined, it is apparently not higher than the percentage found in primates.

SUMMARY

Recent data stress the importance of matching donor and recipient of an organ graft for both the serologically defined (SD) and lymphocyte-defined (LD) determinants. To allow experimental evaluation of the effect of these SD and LD structures in a noninbred experimental animal, mixed leukocyte culture tests were performed between SD identical and nonidentical dogs to clarify the LD system in these animals.

The results of these experiments can be summarized as follows: (a) In the dog there is a LD locus distinct from the known SD loci, which in all probability is localized outside the first (SD-1) series locus on the chromosome. (b) The cross-

ing-over frequency between the SD and LD loci on the chromosome is low. (c) Studies in SD identical unrelated dogs and random unrelated dogs show an apparent high linkage disequilibrium between SD and LD loci. (d) The LD system in dogs is polymorphic.

The authors gratefully acknowledge the excellent technical assistance of Miss Els Blokland, Dienne Elferink, and Els Goulmy for doing the mixed leukocyte culture tests, and Mr. J. Katsbergen and Mr. P. Kreeft for obtaining the blood. We thank Doctors Miriam Segall and James F. Crow for many valuable discussions and Dr. Dick D. Habbema for statistical evaluation.

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