CONSEQUENT INTRAFAMILIAL IMMUNIZATION FOR DL-A HAPLOTYPING IN CANINES*

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(Received for publication 17 October 1972)

The identification by lymphocyte cytotoxicity testing of a polymorphic "H" system of antigens associated with transplantation immunity is a major basis for histocompatibility typing at the present time. Recently the complexity of the histocompatibility chromosomal region has become more evident through studies of the mixed leukocyte reaction and the response of inbred rodent strains to synthetic antigens (1-3).

Although inbred rodent strains have been useful in defining individual genetic factors associated with the immune response, the study of a randomly bred species is essential to understanding the complex human situation. Such studies are only possible when accurate "H" typing is available.

Canines constitute a widely used outbred species for preclinical transplantation studies. Previous investigation in dogs have partially defined the genetics of a DL-A system analogous to HL-A (4). Antisera for typing purposes in both man and dog are produced under two genetic conditions: (a) immunizations between genetically related individuals with one haplotype difference (5, 6); (b) immunizations over a two haplotype difference, e.g., between unrelated individuals (7).

The first method is favored because the number of incompatible haplotypes is only one and, therefore, the number of incompatible antigens is limited. It has been used in inbred mice strains, in man, and to a lesser extent in primates, dogs, and guinea pigs (8-12).

We have not been able to find a study where this immunization method has been perused until all haplotypes of a colony of dogs were recognized. We proposed to call such a procedure consequent intrafamilial immunization. Such immunization has great advantages. By testing the sera in the family in which they are raised, the optimal number of immunizations and dilutions of the reagents can be determined. It can be predicted whether an antibody can be formed and, if so, how the pattern should segregate in the sibs of the family. Much less time is needed to obtain a full set of reagents that can recognize all

* In part supported by the National Institutes of Health (PH43-65-992), the Dutch Organization for Applied Research (TNO), the J. A. Cohen Institute for Radiology and Radiation Protection, and the Whitehall Foundation.

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494 THE JOURNAL OF EXPERIMENTAL MEDICINE • VOLUME 137, 1973
haplotypes than by random immunizations. Selective matings can be carried out and homozygosity for part or all of the "H" region can be identified. This study describes how this procedure enabled us to help clarify the DL-A system. The segregation patterns of the obtained antisera were compared with the results of mixed lymphocyte culture (MLC)\(^1\) tests. The specificities of the obtained antisera were also compared in the general dog population with a panel of antisera produced in most cases by random immunization (4).

**Materials and Methods**

**Dogs.**—The pedigree of the colony studied is depicted in Fig. 1. Of the original parental generation, only bitch B was available for typing. Generation II (F\(_1\)) produced 12 litters with 67 offspring (generation III, F\(_2\)). Random dogs were obtained from local animal handlers. Only mongrels which were unrelated were used.

**Cytotoxicity Typing.**—Typing was done with a modified one-stage microlymphocytotoxicity test (13). Lymphocytes were washed in Earle's balanced salt solution and resuspended in rabbit complement. Preliminary typing was done with a panel of 69 canine alloantisera (4).

**Immunizations.**—Cells for immunization were prepared from 50 ml of heparinized venous blood. Red cells were sedimented with 5% dextran (mol wt 200,000) in isotonic saline, 1 vol dextran to 5 vol of blood. The mixture was incubated for 30 min at 37°C, and the leukocyte-rich supernatant was centrifuged for 10 min at 400 g. The cells were washed twice in isotonic saline and resuspended to a volume of 1–2 ml. Two immunizations, a week apart, were carried out by intradermal injections at four sites. Subsequent intravenous injections of 10 ml of heparinized blood were administered weekly until antibodies against the immunizing donor were demonstrated. The animals were then bled and the sera stored in small aliquots in glass vials at -70°C.

\(^1\)Abbreviation used in this paper: MLC, mixed lymphocyte culture.
INTRAFAMILIAL IMMUNIZATION FOR DL-A HAPLOTYPING

Titrations.—All serial dilutions of antisera were made in inactivated dog serum, which was neither toxic nor potentiating.

Mixed Lymphocyte Cultures.—Dog lymphocytes were isolated on Ficoll-Isopaque gradients as used for serologic typing (13). 40 ml of venous blood were collected in a siliconized bottle, containing 40 ml of Medium 199, buffered with 5 mmol N-2-hydroxyethylpiperazine-N’-2-ethane sulfonic acid (TC199H) (Flow Laboratories, Irvine, Ayrshire, Scotland; no. 1-049), and glass beads. After gently shaking for 10 min the defibrinated blood was layered in 8-ml aliquots on 4 ml of Ficoll-Isopaque and the gradient was centrifuged for 15 min at 1,000 g. The lymphocyte-rich interface was harvested and washed twice in complete medium, TC199H supplemented with 10 mmol t-glutamine, 10^6 U of penicillin/100 ml, and 20% fetal calf serum. This procedure routinely yielded preparations of which 80-90% were lymphocytes. Two-way MLC tests were performed in triplicate in 1-ml vol, and were prepared by mixing equal volumes of cells whose initial concentrations were 2 X 10^6, 1 X 10^6, and 0.5 X 10^6/ml. Such dose response cultures were necessary to determine the optimal concentrations of cells in each experiment. One-way MLC tests were established employing conditions that preliminary experiments indicated to be optimal. The stimulating cells were diluted to between 2 and 6 X 10^6 cells/ml and treated with mitomycin C (A. Christiaens s.a., Brussels) at a final concentration of 1.6 μg/ml at 37°C for 30 min. The cells were then washed with TC199H and resuspended to the desired cell concentration in complete medium. Triplicate 1-ml cultures were established with the responder population at a final concentration of 0.5 X 10^6 cells/ml and the stimulating cells at final concentration of 1 X 10^6, 0.5 X 10^6, and 0.25 X 10^6 cells/ml. Optimal incubation time was found to be 5 days. On day 4, 0.3 μCi of [14C]thymidine (sp act 6.25 mCi/mm) was added. The cultures were harvested on filters and a stimulation index was obtained by dividing the counts per 10 min for the culture (A + B) by the sum of the two cell populations alone, (A) + (B):

\[
\text{Stimulation index} = \frac{(A + B)}{(A) + (B)}.
\]

RESULTS

Table I shows the presumptive haplotyping of the A2 X C3 mating, which was selected for the first intrafamilial immunization. Haplotyping was done on the basis of 10 informative sera from a panel of 69. It should be noted that the haplotyping was somewhat ambiguous, i.e., for C21 and antisera 5 and 9. An immunization scheme (Fig. 2) was used to produce the antisera recognizing

| Cells | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10  | Haplotype |
|-------|----|----|----|----|----|----|----|----|----|-----|-----------|
| A2    | ++ | 1++| 1++| 1++| 1++| 1++| 1++| 1++| 1++| 1++ | a/b       |
| C3    | -  | -  | -  | -  | +  | +  | +  | +  | +  | +   | c/d       |
| C16   | -  | -  | -  | -  | +  | +  | -  | -  | +  | +   | b/d       |
| C18   | ++ | 1++| 1++| 1++| 1++| 1++| 1++| 1++| 1++| 1++ | a/d       |
| C19   | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++   | a/c       |
| C20   | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++   | a/b       |
| C21   | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++   | b/d       |

TABLE I
Presumptive Haplotyping of the Mating A2 X C3 on the Basis of 10 Informative Patterns of Sera Reactions (Backcrosses) from a Panel of 69 Antisera
each of the parental chromosomes. When positive reactions were demonstrated against the immunizing donor cells, the serum was used in titration against the whole family. Table II shows the results in the optimal dilution. It can be seen that all four haplotypes can be assigned unambiguously on the basis of these results. This table clearly demonstrates the mendelian codominant character of the "system." The sera were named after the dogs in which they were produced.

Table II

| Cells | Antisera, with dilution used | Haplotype |
|-------|-----------------------------|-----------|
|       | C21 1/1                     | C19 1/4   | C16 1/2   | A2 1/1 |
| A2    | +++                         | +++       | -         | -      | a/b    |
| C3    | -                           | -         | +        | ++     | c/d    |
| C16   | -                           | +++       | -         | +      | b/d    |
| C18   | +++                         | -         | -         | +      | a/d    |
| C19   | +++                         | -         | +++       | -      | a/c    |
| C20   | +++                         | -         | -         | +      | a/d    |
| C21   | -                           | +++       | -         | +      | b/d    |

The F1 generation C1–C5 were typed with the obtained antisera, as well as the litters they produced. In all litters, the reactions with the two sera, for which the father is positive, appear to segregate and to be mutually exclusive. In contrast, C1 and C2 reacted positive only with serum C16, while some of their offspring were negative with that serum. This in all probability implied that an antigen on one haplotype was recognized by serum C16, while the product of the other haplotype escaped detection. In order to recognize this haplotype one more selective intrafamilial immunization was carried out, and the resulting serum, C32, gives mutually exclusive positive reactions with serum C16, Table III.

Table IV summarizes the typing of the offspring from the C families in the
F₂ generation, N = 57. Observed and expected number of haplotypes show an excellent fit.

To check the validity of the serologic data MLC tests were performed. DL-A-identical dogs C63 and C47 born from different bitches in the colony were tested. Experiments were carried out on 2 separate days. Table V shows the very low stimulating index of the serologically identical dogs as compared with non-identical pairs.

**TABLE III**

*Results of Typing of the A₂ × C₁ Mating with the Sera, Obtained in the A₂ × C₃ and A₂ × C₁ Matings*  

| Cells   | Antisera, with dilution used | Haplotype |
|---------|-----------------------------|-----------|
|         | C₂₁ 1/1                     | C₁₉ 1/4   | C₁₆ 1/2 | A₂ 1/1 | C₂₂ 1/1 |
| A₂      | +++                         | +        | ++      | -      | +++    | a/b |
| C₁      | -                           | -        | +       | +      | -      | c/d |
| C₂₈     | +++                         | -        | ++      | -      | -      | a/c |
| C₃₀     | +++                         | -        | +++     | -      | -      | a/c |
| C₃₂     | -                           | +        | +++     | -      | -      | b/c |
| C₅₀     | +++                         | -        | -       | -      | +     | a/d |
| C₅₁     | +++                         | -        | -       | -      | +     | a/d |

**TABLE IV**

*Observed and Expected Number of Haplotypes of 57 F₂ Offspring of the Colony*

| Haplotype | Observed | Expected | X² |
|-----------|----------|----------|----|
| a         | 37       | 31       | 1.2|
| b         | 23       | 28.5     | 0.9|
| c         | 21       | 26       | 1.0|
| d         | 16       | 13       | 0.7|
| e         | 17       | 15.5     | 0.1|

N = 114; X² = 3.9; 0.30 < P < 0.50.

From the mating A₂ × C₅ (Fig. 1) with the haplotypes a/b and a/d, respectively, the offspring C₃₅ and C₃₇ with the phenotypes a, and a, b were born. As all four haplotypes in the parents could be identified, the possibility was considered that C₃₅ was homozygous for the haplotype a. MLC tests were performed, which fully confirmed this assumption, Table VI.

The reactions with the antisera, obtained by consequent intrafamilial immunizations, were compared with 69 antisera recognizing previously described groups. Table VII shows the X² associations between the antisera and the currently proposed DL groups for both dogs in the colony and for 102 unrelated dogs.
TABLE V
Mixed Lymphocyte Cultures between DL-A-Identical Nonsibling Colony Members C63 and C47

| Haplotype combination | Dog nos. | cpm  | Stimulation index |
|-----------------------|----------|------|-------------------|
| a/c + b/d             | C56 + C63 | 1,297| 19.6              |
|                       | C56 + C47 | 605  | 6.5               |
|                       | C64 + C63 | 1,023| 12.8              |
|                       | C64 + C47 | 1,622| 15.2              |
| b/d + b/d             | C63 + C47 | 121  | 1.2               |

TABLE VI
Confirming Evidence for the Existence of Haplotype Homozygosity in C35

| Haplotype combination | Dog nos. | cpm  | Stimulation index |
|-----------------------|----------|------|-------------------|
| a/b + x/y             | A2 + X   | 2,037| 12.8              |
|                       | C37 + X  | 2,087| 10.1              |
|                       | C35 + X  | 2,314| 18.2              |
| a/(a) + x/y           | A2 + C35 | 1,497| 10.5              |
|                       | C37 + C35| 1,258| 6.6               |
| a/b + a/(a)           | A2 + C37 | 580  | 2.6               |
| a/b + a/b             | C37 + C35m| 542  | 0.8               |
| a/b + a/(a)           | C37m + C35| 1,532| 5.6               |
|                       | A2 + C35m| 557  | 1.3               |
|                       | A2m + C35| 2,074| 7.3               |

m = mitomycin-treated cells.

TABLE VII
Comparison between the Antisera Produced by Consequent Intrafamilial Immunization and Antisera Currently Used to Recognize DL Groups

| Haplotype recognized | Serum donor | Serum no. | DL group recognized | X² of independence in |
|----------------------|-------------|-----------|---------------------|-----------------------|
|                      |             |           |                     | Colony | beagles | mongrels |
| a                    | C21         | 14010     | 1                   | 49     | 5       | 9        |
| b                    | C19         | 14013     | 3                   | 19     | 2       | 4        |
| c                    | C16         | 14009     | 4                   | 35     | 17      | 19       |
| d                    | A2          | 14011     | 2                   | 35     | 7       | 0        |
| e                    | C32         | 14014     | 3                   | 35     | 29      | 24       |

Table VIII shows the serologic data in the A2 × B2 mating. In this mating serum C16 recognizes both maternal haplotypes yielding positive reactions in all siblings. Serum C21 recognizes only one maternal haplotype as indicated by the intercross pattern with this antiserum. Since serum C16 was produced against a
INTRAFAMILIAL IMMUNIZATION FOR DL-A HAPLOTYPING

**TABLE VIII**

*Serologic Data and MLC Confirmation in the A2 × B2 Family*

| Antiserum producer: Immunizing cells: Haplotypes recognized: | B27 | C19 | C11 | C16 | Haplotype designation on basis of Serology alone MLC's added |
|-------------------------------------------------------------|-----|-----|-----|-----|-------------------------------------------------------------|
| A2 | A2 | A2 | C3 | | |
| Immunizing cells: | a, f | c, f | | | |
| a/b | | | | |
| a/c or a/f | a/c | |
| c/f | | | |
| B2 | ++ | ++ | ++ | - | a/b |
| B22 | ++ | ++ | ++ | ++ | a/c or a/f |
| B24 | - | ++ | + | ++ | b/f |
| B26 | - | ++ | - | ++ | b/c |
| B27 | - | + | + | ++ | b/f |
| B28 | - | ++ | + | ++ | b/c |
| B30 | - | ++ | - | ++ | b/c |
| B31 | ++ | ++ | ++ | ++ | a/c or a/f |
| B32 | ++ | ++ | ++ | ++ | b/f |

By consequent intrafamilial immunization reagents were rapidly developed that recognized all haplotypes in this canine colony. Since canines are commonly used for grafting experiments, accurate histocompatibility testing is of crucial importance in evaluating maneuvers designed to alter the immune response. Canine histocompatibility testing has been described using panels of antisera obtained by immunization between random dogs or littermates (6, 4, 14). Although accurate selection of DL-A-identical siblings is successful in most instances, large panels of antisera are required and ambiguous or incomplete family typing is not unusual. The evaluation of such antisera is a prolonged procedure and satisfactory panels may require years to develop (4). In the present study highly accurate serologic reagents were obtained in less than...
In the present study a panel of reagents detecting DL-A antigens were available, providing a basis for selective intrafamilial immunization. This, however, is not an absolute prerequisite. Random immunization of siblings with paternal and maternal cells without preliminary haplotyping should also be effective in developing reagents. The subsequent testing of the family with the reagents produced provides an internal genetic check on the quality of the antisera and optimal conditions for its use. Although planned intrafamilial immunization has been carried out in man (8), for practical reasons this is difficult to do in a complete fashion. It should be noted, that the present studies indicate no evidence of cross-reactivity in the reagents obtained. Theoretically cross-reactivity of antisera against antigens not present in the immunizing donor could interfere with clear-cut recognition of the segregation of the parental chromosomes. The problem of cross-reactivity has been clearly demonstrated in HL-A antisera and represents an unknown variable in interpreting data in the DL-A system (15). The apparent lack of cross-reactivity with the present reagents may be due to the cessation of immunization when clear-cut positive results with the immunizing donor were obtained. In addition the antisera were only used in dilutions which gave the optimal segregation patterns.

These studies revealed that consequent intrafamilial immunization is an excellent technique for identifying homozygous individuals in a closed colony. This was confirmed by one-way MLC in the appropriate serologic setting. The ability to select homozygous individuals for experimental transplantation studies has obvious advantages. The precise H typing described provides a basis for rapid identification of crossing-over and formal proof of a two loci DL-A system. In 67 offspring of the F₂ generation of the colony crossing-over was not detected. However, the serologic reactions in the A₂ × B₂ mating do provide additional support for the population analysis of Vriesendorp et al. indicating at least two subloci in the DL-A system (4).

The accurate H typing of this colony will make possible investigations for the presence of an immune response locus linked to, but separable from DL-A. Canines would seem ideal for such studies in a large, randomly bred species. In man increasing evidence indicates the existence of an MLC locus outside of the HL-A region (1). These findings may be of considerable importance when unrelated HL-A identical donor-recipient pairs are used in tissue grafting experiments (16). As previously demonstrated in man and dogs, H typing correlates well with MLC reactivity in sibships (17, 18). The present studies extends these observations to members of a canine colony that are not of the same litter.

SUMMARY

A procedure of intrafamilial immunization is described for production of antisera recognizing DL-A haplotypes. In a colony consisting of 1 sire, 6 bitches,
and 67 offspring all haplotypes could be accurately allocated. In the colony the observed reaction frequencies of the antisera are in agreement with mendelian codominant inheritance. Mixed lymphocyte culture tests confirmed the accuracy of the serologic typing and the presence of homozygous individuals within the colony. Further evidence is presented supporting the presence of two or more subloci within the DL-A system. Colonies of canines such as the one described should provide a sensitive system for evaluating interaction between serologic DL-A typing, MLC reactivity, and immune response genetics in a nonrodent species which is not highly inbred.

The dog colony used in these studies is maintained by a grant from the Dutch Organization for Fundamental Medical Research (FUNGO) to Dr. J. J. Veltkamp. The serology was done by Miss G. van Lottum and Mrs. G. Zwiers-Oostergo. The skillful animal handling of Mr. van der Zweet and Mr. Mathot is gratefully mentioned. Miss Hiensch assisted with the MLC testing.

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