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Class I and Class II Regions of the Major Histocompatibility Complex Both Contribute to Individual Odors in Congenic Inbred Strains of Rats

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The major histocompatibility complex (MHC) of the rat has three regions—A (class I), B/D (class II), and C/E (class I)—and congenic strains are available which differ in each of these regions. We used the habituation–dishabituation procedure to examine the ability of PVG-RT1^u male rats to discriminate between the urinary odors of congenic rat strains which differ genetically only at certain individual regions of the MHC. The results of five experiments indicate that discrimination can be made between urine from rats which differ in all three regions of the MHC (PVG vs. PVG-RT1^{av1} donors), only in the class I A region (PVG vs. PVG.R1 donors), only in the class I C/E region (PVG.R19 vs. PVG-RT1^{av1} donors), only in the class II B/D region (PVG.R1 vs. PVG.R19 donors), and in all regions except the classical class IA locus (PVG-RT1^{av1} vs. PVG.R1 donors). These results indicate that all of the MHC regions may contribute to the individual odors of rats.

KEY WORDS: major histocompatibility complex; olfaction; individual odors, habituation; chemosensory identification; rats; *Rattus norvegicus*; congenic strains.

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INTRODUCTION

The major histocompatibility complex (MHC) is a linked cluster of genes which code for structural glycoproteins inserted into the cell membranes. These membrane molecules carry the major histocompatibility antigens which are responsible for the rapid rejection of tissues transplanted from one individual to another in nearly every species so far examined. The MHC genes are two major types. The class I genes, which are expressed in virtually every cell of the body, encode a 45-kd polypeptide associated noncovalently on the cell surface with a 13-kd protein (β2 microglobulin) which is not encoded by the MHC. The class II genes encode a dimeric molecule expressed predominantly on the surface of certain cells of the immune system (Hood *et al.*, 1983).

Although class I and class II MHC glycoproteins were originally defined as the agents which induce rejection of grafts exchanged between individuals within a species, they also function within an individual organism to present processed antigens for recognition by the T cells to trigger the immune response. Class I molecules present antigen to the cytotoxic T cells, while class II molecules present antigens to the helper cells which promote antibody synthesis by B cells. Recent structural studies have identified the antigen-binding cleft on the surface of class I molecules (Bjorkman, *et al.*, 1987a,b). Receptors on T cells recognize the composite structure consisting of the bound antigen fragments and the surrounding variable determinants of the MHC itself.

The class I molecules are of two types: the so-called classical and nonclassical antigens. The classical class I molecules are expressed on all or nearly all cells in the body and are extremely polymorphic within a species, there being more than 100 alleles at each of three genetic loci in man and similar allele frequencies in other species (Klein, 1986). The nonclassical class I antigens, although they have an overall structure similar to that of the classical molecules are oligomorphic (for some of these antigens there is only a single allele within a species), occupy many more loci over a much larger stretch of DNA, and have a restricted tissue distribution (Hood *et al.*, 1983).

The genetic loci that encode these molecules in both mouse and rat are comparable (Brown *et al.*, 1988). The three regions of the rat MHC, termed RT1, are RT1A, RT1 B/D, and RT1 C/E, and these correspond to the mouse H-2 regions, H-2K, H-2I (A/E), and probably D/Qa:T1a (Butcher and Howard, 1986). In the mouse, H-2K and H-2D encode classical class I molecules, H-2I encodes class II molecules, and Qa:T1a encodes nonclassical class I molecules. So far, no recombination has separated the C region in the rat MHC from a region analogous to the mouse

Qa:T1a region. Functionally, the gene products of the C region show some of the properties of classical class I molecules, for example, the ability genetically to restrict cytotoxic responses to a minor transplantation antigen (Livingstone, 1983), but they are a much weaker barrier to transplantation than the classical polymorphic class I antigens of the A region.

The individual odor of an animal has been linked to individual differences in the MHC (Brown, 1983; Beauchamp *et al.*, 1986; Boyse *et al.*, 1987; Yamazaki *et al.*, 1980). In mice, different class I regions of the MHC contribute to the odors which can be used to discriminate between congenic strains (Yamazaki *et al.*, 1979, 1982, 1983; Yamaguchi *et al.*, 1981). Yamazaki *et al.*, (1989) have recently shown that the class II region of the mouse MHC also influences the production of unique urinary odours.

In previous papers (Brown *et al.*, 1987; Singh *et al.*, 1987, 1988a), we have shown that two strains of rats (PVG and PVG.R1) which differ only in the classical class I region (RT1A) of the MHC produce discriminably different urinary odors. In this paper, we describe a series of five experiments which examine the ability of rats to discriminate between the urine odors of congenic strains of rats which differ in (1) all three RT1 regions of the MHC (PVG versus PVG-$RT1^{av1}$), (2) only the classical class I region RT1A (PVG versus PVG.R1), (3) only in the nonclassical class I RT1 C/E region (PVG.R19 versus PVG-$RT1^{av1}$); (4) only in the class II RT1 B/D region (RVG.R1 versus PVG.R19), and (5) in all MHC regions except the classical class I RT1A region (PVG-$RT1^{av1}$ versus PVG.R1). These comparisons are summarized in Table I.

EXPERIMENT 1

In this experiment, we examined the distinctiveness of urinary odors from congenic strains of rats which differed in all three MHC regions (PVG versus PVG-$RT1^{av1}$).

Methods

Subjects. The test subjects were 32 male PVG-$RT1^{u}$ rats born in the specific pathogen-free (SPF) unit at Babraham and housed in groups of two or three in 23.2 × 35.2 × 18-cm plastic cages with wood shavings for bedding. These rats were kept on a reversed 12:12 L:D cycle with lights out at 8:00 AM and received ad lib. water and food (Labsure irradiated diet CRM NUTS). They were tested when 84–112 days of age

Table I. Differences in Congenic Rat Strains at Each Region of the MHC (RT1) on Chromosome 14 (Oikawa *et al.*, 1984)

Congenic strain	MHC region		
	Class I A	Class II B/D	Class I C/E
Experiment 1. Two strains which differ in all MHC regions			
PVG (P)	*c*	*c*	*c*
PVG-*RT1*^{avl} (A)	*avl*	*avl*	*avl*
Experiment 2. Two strains which differ only in the classical class IA region			
PVG (P)	*c*	*c*	*c*
PVG.R1 (R)	*avl*	*c*	*c*
Experiment 3. Two strains which differ in the class I C/E region			
PVG.R19 (R19)	*avl*	*avl*	*c*
PVG-*RT1*^{avl} (A)	*avl*	*avl*	*avl*
Experiment 4. Two strains which differ only in the class II B/D region			
PVG.R1 (R)	*avl*	*c*	*c*
PVG.R19 (R19)	*avl*	*avl*	*c*
Experiment 5. Two strains which differ in all MHC regions except the classical class I A region			
PVG.*RT1*^{avl} (A)	*avl*	*avl*	*avl*
PVG.R1 (R)	*avl*	*c*	*c*

and all tests were carried out in the dark phase of the L:D cycle under dim white light illumination.

Urine Donors. Urine was collected individually from eight PVG (P) and eight PVG-*RT1*^{avl} (A) males which were about 150 days of age. These rats were placed in Urimax metabolism cages in the Babraham SPF unit, and when the urine was collected, it was frozen at −20°C until used in the tests. Pools were made from equal quantities of urine from each of four males, so that there were two pools from each strain of rats.

Apparatus and Procedure. Habituation–dishabituation tests were conducted in a test arena made from a 29.6 × 23.6 × 14.6-cm opaque animal housing cage with a wire-mesh top extending up another 5.6 cm, making the cage 20.2 cm tall (Brown *et al.*, 1987).

Odors were presented by placing 0.1 ml of liquid onto a 7-cm circle of filter paper (Whatman No. 1) taped to one side of the cage so that the

center of the filter paper was about 13.5 cm from the floor. A stopwatch was used to record the time that the subjects spent rearing and sniffing the filter paper. Each odor presentation lasted 2 min, after which the top of the cage was removed and replaced with another top containing a new piece of odorized filter paper. The used top was then washed with a 70% alcohol solution and allowed to dry before being used again.

Each subject received nine 2-min odor presentations, the first three presentations with water on the filter paper, followed by three presentations of urine sample 1 and three with urine sample 2. Sixteen subjects were tested with urine samples from the two different strains, eight in the order P–A and eight in the reverse order. Another 16 subjects were tested with two samples of urine from different pools of the same strain, eight with two samples of urine from PVG-$RT1^{av1}$ (A) males and eight with two samples of urine from PVG (P) males.

Statistical Analysis. A randomized-blocks ANOVA was conducted over the nine trials for each group of eight subjects and Newman–Keuls post hoc tests were conducted to investigate differences among the means following a significant overall F test (Kirk, 1968).

Results and Discussion

When urine from the two different strains of rats was presented (Fig. 1a), there was a significant difference in time rearing and sniffing the odors over the nine presentations in both test order sequences [P–A; $F(8,56) = 16.83$, $p < .001$; A–P; $F(8,56) = 12.84$, $p < .001$], with the highest means on trials 4 and 7 ($p < .01$).

When two pools of urine from the same strains (A–A or P–P) were presented (Fig. 1b), there was a significant difference in investigation time over the nine trials [A–A; $F(8,56) = 19.92$, $p < .001$; P–P; $F(8,56) = 13.32$, $p < .001$], but only trial 4 differed from the other trials ($p < .01$).

Thus, males of the PVG and PVG-$RT1^{av1}$ strains, which differ in all three MHC areas, produce urinary odors which are readily discriminable.

EXPERIMENT 2

We have previously shown that two strains of congenic rats which differ only in the classical class IA region of the MHC (PVG versus PVG.R1) can be discriminated by their urine odors (Brown *et al.*, 1987; Singh *et al.*, 1987, 1988a). This experiment repeats this test using pools of urine rather than urine from individual donors.

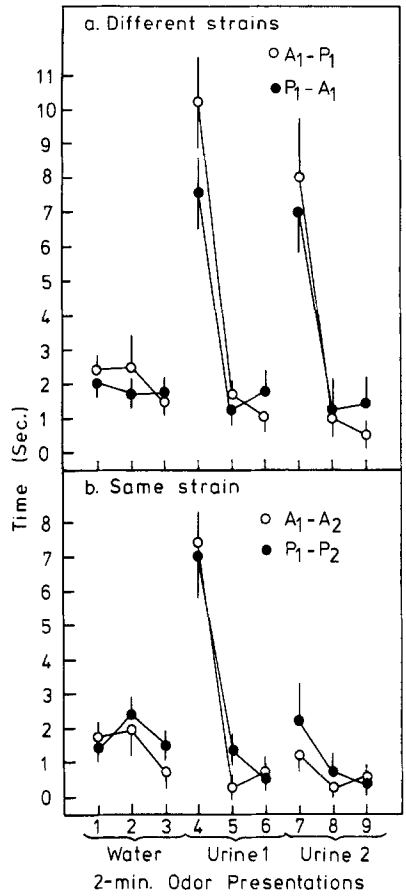

Fig. 1. Mean time (±SE) spent rearing and sniffing at urine odors from (a) PVG-*RT1*^{av1} (A) and PVG (P) donors and (b) two pools of urine collected from individuals of the same strain.

Methods

Subjects. Twenty-four male PVG-*RT1*^{u} rats were used as subjects in this experiment; 8 were born in the SPF unit at Babraham and 16 were purchased from the Pathology Department at the University of Manchester Medical School. These subjects were housed under the conditions described under Experiment 1.

Urine Donors. Urine was collected from eight PVG (P) and eight PVG.R1 (R) males in Urimax metabolism cages and frozen at −20°C until

used. Two pools of urine were produced for each donor strain of rat by pooling equal quantities of urine from each of four individual males.

Apparatus and Procedure. The apparatus and procedure described under Experiment 1 were used. Sixteen males were tested with urine from different strains, eight in the order P–R and eight in the reverse order. A third group of eight males was tested with two pools of urine from PVG.R1 males. Separate tests were not done on urine from two pools of PVG urine as these are described under Experiment 1.

Results and Discussion

As shown in Fig. 2a, there were significant differences in the time spent investigating urine odors over the nine presentations for both test sequences [P–R; $F(8,56) = 32.58$, $p < .001$; R–P; $F(8,56) = 20.60$, $p < .001$], with more time spent on trials 4 and 7 than on any other odor presentations ($p < .01$).

When two pools of urine from the PVG.R1 strain were presented, there was a significant difference in the time spent sniffing over the nine presentations [$F(8,56) = 31.80$, $p < .001$], with more time spent sniffing on trial 4 than on any other trial ($p < .01$).

This experiment confirms our previous findings (Brown *et al.*, 1987; Singh *et al.*, 1987; 1988a) that differences in the classical class I region RT1A of the MHC result in strains with discriminably different urinary odors.

EXPERIMENT 3

In this experiment, we examined differences in urine odors from congenic strains of rats which differ only in the class I C/E region of the MHC (PVG.R19 and PVG-$RT1^{av1}$; see Table I).

Methods

Subjects. Twenty-four male PVG-$RT1^u$ male rats were used as subjects. These males were purchased from the Pathology Laboratory at the University of Manchester when 112–161 days of age and housed at Babraham under the conditions described under Experiment 1.

Urine Donors. Urine was collected in Urimax metabolism cages from eight male PVG.R19 (R19) and eight male PVG-$RT1^{av1}$ (A) male rats which were about 150 days of age. Two pools of urine were made from each strain by combining equal amounts of urine from four individual donors.

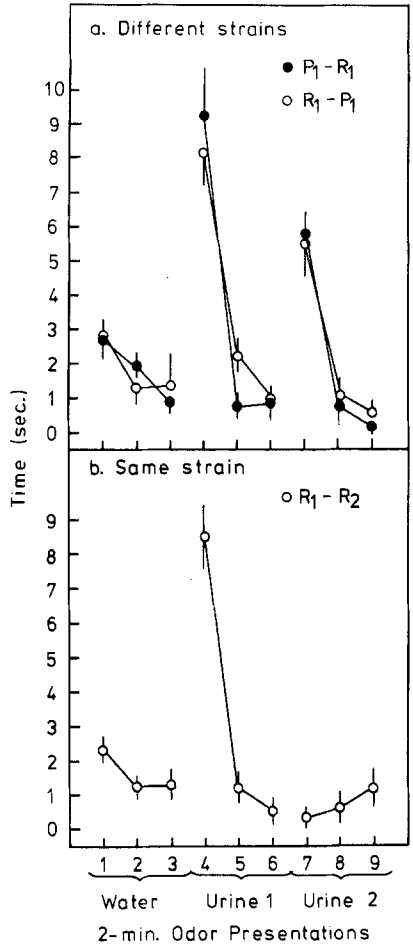

Fig. 2. Mean time (±SE) spent rearing and sniffing at urine odors from (a) PVG (P) and PVG.R1 (R) donors and (b) two different pools of urine collected from individuals of the PVG.R1 strain.

Apparatus and Procedure. The test apparatus and procedure were the same as described for experiment 1. Eight subjects were tested in the order R19–A and eight in the reverse order. The remaining eight were tested with two different pools of urine from the PVG.R19 donors.

Results and Discussion

There was a significant difference in time investigating urine odors over the nine presentations for males tested with urine from two different

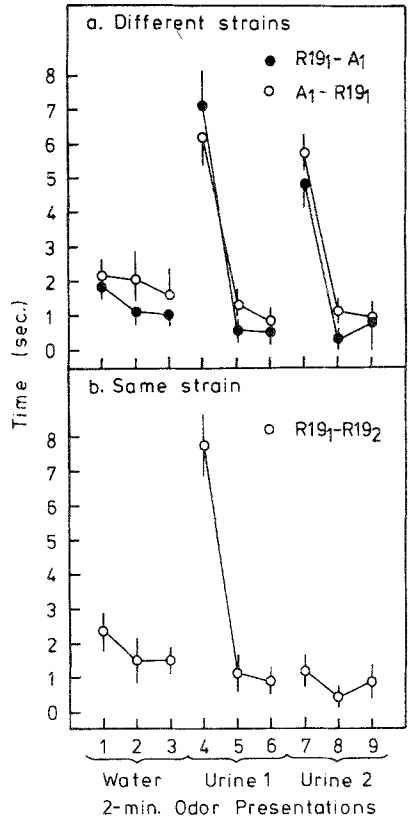

Fig. 3. Mean time (±SE) spent rearing and sniffing at urine odors from (a) PVG-*RT1*^{av1} (A) and PVG.R19 (R19) donors and (b) two pools of urine collected from individuals of the PVG.R19 strain.

strains of donors for both orders of presentation [R19–A; $F(8,56) = 21.86$, $p < .001$; A–R19; $F(8,56) = 13.93$, $p < .001$], with more time spent investigating urine on trials 4 and 7 than any other trials for both groups of subjects ($p < .01$) (Fig. 3a).

When urine was from two pools of R19 donors, there was an overall difference in the time spent investigating over all nine trials [$F(8,56) = 19.15$, $p < .001$] and more time was spent rearing and sniffing on trial 4 than on any other trial ($p < .01$). The results from the two pools of urine from PVG-*RT1*^{av1} rats are the same (Fig. 3b).

It is clear from these results that two congenic strains of rats which differ only in the class I C/E region are able to produce discriminably

different urine odors, indicating that this region of the MHC may contribute to the uniqueness of individual odors as well as the class I A region.

EXPERIMENT 4

This experiment examined differences in urinary odors between male PVG.R1 and male PVG.R19 rats, congenic strains which differ only in the class II B/D region of the MHC (Table I).

Methods

Subjects. The subjects were 16 male PVG-*RT1*^{u} rats purchased from the Pathology Laboratory of Manchester University Medical School at 80–110 days of age. They were housed under the conditions described under Experiment 1.

Urine Donors. Urine from eight male PVG.R1 (R) rats was made into two pools from four males each as was the urine from the eight PVG.R19 (R19) males. The urine donors were the same as those used in experiments 2 and 3, but the pools were different: donors 1, 2, 7, and 8 formed one pool, while donors 3, 4, 5, and 6 formed the other. In experiments 2 and 3, urine pools were from donors 1–4 and 5–8, respectively.

Apparatus and Procedure. The test apparatus and procedure were the same as described under Experiment 1. Eight subjects were tested with urine samples in the order R–R19 and eight in the opposite order.

Results and Discussion

As shown in Fig. 4, there was a significant difference in the time spent investigating urine odors over the nine presentations for both odor presentation sequences [R–R19; $F(8,56) = 18.06$, $p < .001$; R19–R; $F(8,56) = 22.56$, $p < .001$], and in both sequences more time was spent investigating urine on tests 4 and 7 than on any other presentation ($p < .01$).

Two pools of PVG.R1 urine were not discriminable (Fig. 2), nor were two pools of PVG.R19 urine (Fig. 3).

These results indicate that the class II B/D region of the MHC as well as the class IA and C/E regions may contribute to the production of discriminable differences in urine odors of rats.

The two strains PVG.R1 and PVG.R19 differ in the class II region (c versus a), which also contains a modifying gene for class I. Thus, although the class IA regions of these strains are the same type (a), there

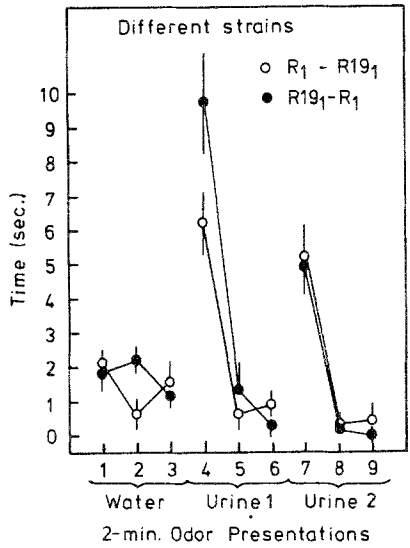

Fig. 4. Mean time (±SE) spent rearing and sniffing at urine odors from PVG.R1 (R) and PVG.R19 (R19) donors.

are two differences in the molecules expressed. There is a quantitative difference in the antigen produced (Howard *et al.*, 1978), and there is a qualitative difference in response to cytotoxic T loci which is determined by the modifying gene in the class II B/D locus (Livingstone *et al.*, 1983).

EXPERIMENT 5

In the final experiment, we examined the difference between urine odors from PVG-*RT1*^{av1} and PVG.R1 males, two congenic strains which are the same in the classical class IA region but differ in the other MHC regions (class II B/D and class I C/E; see Table I).

Methods

Subjects. The subjects were 16 male PVG.*RT1*^{u} rats purchased from the Pathology Laboratory at Manchester University Medical School when 80–110 days of age. They were housed as described under Experiment 1.

Urine Donors. Urine was collected from eight male PVG-*RT1*^{av1} (A) and eight male PVG-R1 (R) rats and two pools of urine were made for each strain by combining equal amounts of urine from four males in each pool.

Apparatus and Procedure. The test apparatus and procedure were the same as described under Experiment 1. Eight males were tested with urine in the order A–R and eight in the reverse order.

Results and Discussion

As shown in Fig. 5, there were differences in the time spent investigating urine odors over the nine presentations for both sequences of testing [R–A; $F(8,56) = 19.74, p < .001$; A–R; $F(8,56) = 20.77, p < .001$] and there was more time spent sniffing on trials 4 and 7 than on any other trial ($p < .01$). Although the response to the second odor (trial 7) was very small for the test order R–A (Fig. 5), the difference between trial 7 and trial 6 was significant at the .01 level because of the low variability in the data.

The two pools of urine from the PVG.R1 males were not discriminably different (Fig. 2), nor were the two pools of urine from the PVG-$RT1^{av1}$ males (Fig. 1).

These results indicate that rats which differ in the class II B/D and the class I C/E regions of the MHC but do not differ in the classical class I A region still produce discriminably different urinary odors.

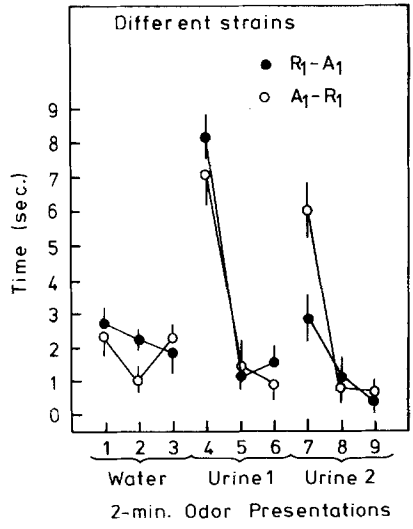

Fig. 5. Mean time (± SE) spent rearing and sniffing at urine odors from PVG.R1 (R) and PVG-$RT1^{av1}$ (A) donors.

GENERAL DISCUSSION

We have previously shown that the classical class I A antigens are secreted in a soluble form into the blood and excreted in a degraded form in the urine. We postulated that this process gives rise to volatile components which are unique to each strain (Brown *et al.*, 1987; 1988; Singh *et al.*, 1987, 1988a). It now appears that other areas of the MHC may also influence these volatile components.

The results presented in this paper confirm those found in the mouse (Yamazaki et al., 1982) that both ends of the MHC, RT1 A (H-2K like) and RT1 C/E (which are thought to contain Qa:T1a-like class I genes) (Butcher and Howard, 1986), are important in confering a scent on an individual. It is now shown that such an ability also resides with the class II loci (Fig. 4). Both classical class I and class II molecules are known to act as restricting elements in antigen presentation (Klein, 1986), suggesting that the mechanism that underlies their ability to confer a characteristic scent on an individual may reside in this common function.

It has been suggested that the *immune-response* (*Ir*) gene profile of an individual, determined by the constellation of alleles at both the classical class I and the class II loci, gives rise to a mixture of commensal bacterial flora peculiar to that individual, which in turn gives rise to the MHC-related odor (Howard, 1977). However, two observations mitigate against this idea. Yamazaki *et al.* (1985) found that in radiation chimeras of the F_1 into the parent type, urinary odors are of the F_1 donor. Since it has been shown that class I-restricted immune responsiveness is heavily biased toward the parental MHC type (Bevan and Fink, 1978; Zinkernagal and Doherty, 1979), it would appear that the immune-response phenotype remains parental in these animals. In addition, it has been shown that mice (Yamazaki *et al.*, 1982) and rats (Fig. 3) that are congenic with respect to the nonclassical Qa:T1a class I loci (the class I C/E region in rats) can be distinguished by odor alone. These molecules have no known *Ir* gene function, although they are found in the circulation of both mice (Maloy *et al.*, 1984; Kress *et al.*, 1983) and rats (Spencer and Fabre, 1987). Bacterial flora do, however, play an important role in the production of the MHC-related odor, as we have shown that germ-free rats fail to produce an odor of individuality (Singh *et al*, 1989).

Soluble classical class I molecules are found in the urine and we have postulated that either degraded fragments of these molecules or volatiles associated in an allele-specific manner with these glycoproteins are responsible for the class I-associated odor (Brown *et al.*, 1987, 1988; Singh *et al.*, 1987, 1988a). At present, no evidence exists for the presence of class II molecules in the body fluids of rats, although they are present in mice (Callahan *et al.*, 1976; Parish *et al.*, 1976).

More recently, the X-ray crystallography of the HLA-A2 class I molecule derived from the tumor line JY (Bjorkman *et al.*, 1987a,b) has shown that these molecules have cocrystallized with smaller molecules that may be peptides. Should some of these peptides be derived from class II molecules, it would provide a unifying mechanism where the class I molecules act as a carrier for the class II-specific molecules which, by a method analogous to that previously proposed (Singh *et al.*, 1987, 1988a), provide a class II-specific scent on an individual.

The MHC is not the only genetic region known to be involved in conferring a scent on an individual. The genome as a whole, apart from the MHC, is as potent as the H-2 region as a source of odor individuality, as determined by testing trained rats and mice (Boyse *et al.*, 1987; H. Duncan, personal communication, October 1987). Yamazaki *et al.*, (1986) have also shown that mice differing genetically only in the X and/or Y chromosomes can be discriminated by their odors, thus identifying another genetic locus that may contribute to the individuality of scent. Odors from mice which are congenic with respect to the autosomal loci in the Lyb-2:Mup-1 region (chromosome 4) and the Lyt-2:Lyt-3:IgK region, (chromosome 6) have given inconclusive results when testing by the Y-maze technique (Yamazaki *et al.*, 1986). The possible influence of other autosomal loci in conferring a characteristic scent on an individual is not known in the rat.

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