INHERITANCE OF ANTIBODY SPECIFICITY

I. Anti-(4-Hydroxy-3-Nitrophenyl)Acetyl of the Mouse Primary Response

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At least two types of gene loci are known to control specific antibody responses. One type of gene is usually linked to the major histocompatibility locus (1) and controls antigen recognition by thymus-derived (T) lymphocytes (2–4). Genes of the other type are often linked to the allotype of the immunoglobulin heavy (H)1 chains (5–9). They are probably “V genes,” structural genes of the variable (V) regions of immunoglobulin molecules. Criteria suggesting that a gene controlling a specific response is a V gene include (a) allotype linkage; (b) that its effect is limited to only one determinant (often an artificial hapten) of the whole molecule, being independent of the other parts of the antigen (the carrier); (c) that it affects specificity rather than quantity of an antibody; (d) that it controls an idiotypic of a specific antibody; and (e) it controls antibodies of restricted isoelectric patterns (10, 11).

Several reports have been published about genetically controlled immune responses of mice where V genes seem to be involved. Two methods have been used, the study of idiotypes (5–9) and of the fine specificity of antibodies (12). The possibilities of the former method are somewhat limited by the fact that simply inherited idiotypes occur mainly in certain antigen-strain combinations (arsanilic acid-A/J [7], phosphorylcholin-BALB/c [5], and streptococcal carbohydrate-A/J [8]) while most antigen-strain combinations produce individual rather than strain-specific idiotypes. More strain-specific idiotypes could perhaps be found by using cross-reactive rather than identical antibodies for raising the anti-idiotypic and as proband in the inhibition test (13).

Fine specificity characteristics were first studied by Paul et al. (12), who found inheritable differences between anti-2,4-dinitrophenyl(DNP) antibodies of vari-

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Abbreviations used in this paper: ABC, antigen-binding capacity; BSA, bovine serum albumin; CG, chicken globulin; H, heavy; HPI, haptenated phage inactivation; HPII, haptenated phage inactivation inhibition; I₅₀, concentration of hapten causing 50% inhibition of a serological reaction; 2-ME, 2-mercaptoethanol; NIP, (4-hydroxy-5-iodo-3-nitro-phenyl)acetyl; NNP, (4-hydroxy-3,5-dinitrophenyl)acetyl; NP, (4-hydroxy-3-nitrophenyl)acetyl; PFC, hemolytic plaque forming cells; amino-cap, N α-amino-n-caproic acid; RI, recombinant inbred; V, variable.
ous mouse strains. We found similar differences between anti-4-hydroxy-3-nitrophenylacetyl (NP) antibodies of different mouse strains (14). C57BL/6 mice consistently produced heteroclitic (15) 7S anti-NP antibodies, they had 3-10 times higher affinity for 4-hydroxy-5-iodo-nitrophenylacetyl (NIP) than for NP, and also higher affinity for 4-hydroxy-3,5-dinitrophenylacetyl (NNP) than for NP. Moreover, the antibody of this strain exhibited a constant affinity and constant cross-reactions at both early and late stages of immunization while anti-NP antibodies produced by the CBA strain were slightly heteroclitic at the beginning of the response but underwent maturation (progressively increased affinity for NP and increased discrimination between NP on one hand and NIP on the other). BALB/c, C3H, DBA/2, and IAH were found to resemble CBA while LP/J and RF/J resembled C57BL/6 (14, and unpublished observations).

In an attempt to elucidate the inheritance of this polymorphism we produced various types of crosses between strains C57BL/6 and CBA. The results are indicative of linked genetic transmission of the genes coding for the fine specificity characteristic of anti-NP antibodies and the H-chain C-region allotype.

Materials and Methods

Inbred, Congenic, and Recombinant Mouse Strains. CBA, C57BL/6, and BALB/c mice were from our colony. C57BL/10Sn were a gift from Dr. Klaus Rajewsky, Department of Genetics, Köln University, Köln, West Germany. The congenic strains B10.A, B10.Br, and B10.D2, and the Bailey recombinant inbred (RI) strains C x BD, C x BE, C x BG, C x BH, C x BI, C x Bj, and C x BK were obtained from the Jackson Laboratories, Bar Harbor, Maine. The inbred strain C57BL/Ka and congenic CB20 were kindly given to us by Dr. Mike Potter, National Cancer Institute, Bethesda, Md.

The Bailey RI strains were derived from seven different pairs of (C57BL/6 x BALB/c)F₂ mice (16). The congenic strains B10.A, B10.Br, and B10.D2 have various H-2 alleles in C57BL/10Sn background (See Fig. 5). The CB20 congenic mice have the IgC₄₅ gene complex from the C57BL/Ka in a BALB/c background (9).

Haptens and Their Conjugates. The haptens used, NP, NIP, NNP, NP-azide, and NNP-azide, were prepared according to the method of Brownstone et al. (17). NIP-azide was prepared according to Hatcher and Miikelä (18). They were conjugated to proteins and erythrocytes via the azides as previously described (14).

Immunization of Mice. The immunogens used were NP coupled to chicken globulin (CG) (protein precipitated by 45% (NH₄)₂ saturation of chicken serum at +4°C pH 7.0) or bovine serum albumin (BSA) (Cohn fraction V, obtained from Armour Laboratories Eastbourne, England). NP₂₀CG contained 15 mol of NP/150.000 g of CG, and NP₂₀BSA contained 14 mol of NP/mol of BSA. Alum-precipitated NP₂₀CG (100 μg/mouse/injection) was injected intraperitoneally with (first injection) or without (second injection) 10⁹ Bordetella pertussis bacteria as adjuvant. NP₂₀BSA (100 μg) was injected subcutaneously (four sites) in complete Freund’s adjuvant. The second injection was administered 40 days after the first. Plaque-forming cells were tested 7 days after the first or the second injection and the serum antibodies were tested 17 days (primary) or 16 days (secondary) after immunization.

Antibody Assays. The amount of antibody was measured by three methods, hemolytic plaque-forming cell number (PFC), radioactive antigen-binding capacity (ABC) (18), and haptenated bacteriophage inactivation (HPI). All these methods have been described (14). In this study we considered only the IgG antibodies. Consequently 2-mercaptoethanol (2-ME) (0.1 M) was used in the HPI test, a mixture of rabbit antirabbit IgM and antirabbit IgG was used in the PFC test, and the ABC test was applied only to study the secondary response.
Affinity and Specificity Assays. Affinity of the antibodies was estimated by inhibiting the above antibody reactions by varying concentrations of the hapten. We interpolated the hapten concentration that caused 50% inhibition of the number of PFC or ABC (I_{50}). In the case of HPI, 50% inhibition was defined as the increase of phage survivors to the level that was found with twice-diluted antibody; the inhibition test is called the haptenated phage inactivation inhibition (HPII) test in the following (see Fig. 3). Fine specificity of an antibody was studied by calculating K_{rel} values according to Pauling et al. (19),

\[ K_{rel} = \frac{I_{se} \text{ for homologous hapten}}{I_{so} \text{ for the related hapten}}. \]

All the methods used for affinity and specificity measurements have been described (14).

Antibody Sera. Anti-Ig-1^{b} was obtained by immunizing BALB/c mice with *B. pertussis* bacteria coated with C57BL/6 antibodies according to Dresser and Wortis (20). Anti-Ig-1^{b} was prepared by immunizing C57BL/6 mice with *B. pertussis* bacteria coated with BALB/c antibodies. The typing was done by double-diffusion method. Our first backcross mice were kindly typed by Dr. Ethel Jacobson, Basel Institute for Immunology, Basel, Switzerland.

Results

Nature of the Strain Characteristics. The general method for studying fine specificity was inhibition of antibody reactions by three related haptens including the immunogenic NP. The concentration causing 50% inhibition of a reaction (I_{50}) (number of PFC, inactivation of NP bacteriophage, or antigen binding) was determined for each hapten, and relative association constants (K_{rel}) were determined. Plotting of these values is illustrated in Figs. 1–3. Reasonably constant and accurate I_{so} values could be obtained by using half-log steps in the concentration series by both the PFC and the HPII method. The antigen-binding method yielded less smooth curves and it was used only for a limited number of sera.

The characteristics of the C57BL/6 and CBA strains emerge from Figs. 1–3 (individual mice) and Tables I–III (mean values). They are expressed in a slightly different manner in the primary and in the secondary responses but the general rule is the same: C57BL/6 7S antibodies of the secondary response had a higher affinity for NIP and NNP than for NP (K_{rel} values were higher than 1 regardless of the method) while anti-NP of the CBA mice behaved in a conventional way (K_{rel} values ≤ 0.15 regardless of the method). The three methods yielded remarkably different I_{so} values, varying within an almost 1,000-fold range. This could be due to different degrees of dependence on multivalent binding in the different tests. (If bivalent binding to NP erythrocytes but monovalent binding to NP bacteriophage was required higher I_{so} values would be expected for the former.) On the other hand different tests can have different affinity thresholds. (If the threshold is much higher for the HPII test than for the PFC test lower I_{so} values should be expected for the former.) For this study, however, it was sufficient that all three methods yielded similar K_{rel} values (0.021–0.15 in CBA, and 1.2–10 in C57BL/6).

In the primary response all I_{so} values were higher than in the secondary response. Anti-NP of the CBA mice did not discriminate as sharply between the
imunogenic NP and its relatives as the antibodies of the secondary response but the strain differences demonstrated by relative affinities were again approximately 30-fold.

CG was the carrier molecule for NP in most of these experiments. We did a limited experiment using NP-BSA as the immunogen. The response was much weaker than the response to NP-CG (log titer HPI ca. 4 instead of ca. 6 [14]) but the CBA and C57BL/6 strain characteristics were present in the anti-NP that was produced (Table III).

Data in Figs. 1-3 and Tables I-III show that strain differences could be demonstrated by any of the three methods tested. We had to select one of them for testing and characterizing larger numbers of mice. The PFC test was rejected because it is laborious and it requires killing of the mice. The HPII test was considered better than the antigen-binding test as it requires only 1/100-1/1000 of the antibody required for the antigen-binding test.

$I_{50}$ values of all three haptens were considered useful but this resulted in two $K_{rel}$ values, NNP aminocap relative to NP aminocap and NIP aminocap relative to NP aminocap. As we wanted to present the data in a two-dimensional...
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**Fig. 2.** Inhibition of secondary response indirect (Ig-G) NP plaques by NP aminocap, NIP aminocap, or NNP aminocap. Spleen cells from CBA, C57BL/6, and (CBA x C57BL/6)F1, were collected 7 days after the second injection of NP-CG. Two individual mice from each strain are represented by black or white circles.

Histogram we decided to calculate the (log) mean of the two $K_{rel}$ values of each animal and use the mean $K_{rel}$ as a single characterization of individual mice.

**Inheritance of the Strain Characteristics.** Mean $K_{rel}$ of 40 CBA and 27 C57BL/6 primary response anti-NP sera were distributed as two nonoverlapping populations. (Fig. 4 A and B). As these distributions were reasonably log normal we calculated the log means. They were 0.23 for CBA and 9.1 for C57BL/6. The gap between the two distributions made a satisfactory basis for testing crosses between two strains. Frequency distribution of 48 F1 mice tested (Fig. 4 C) suggested dominance of C57BL/6 characteristic but there were five mice resembling the recessive parent. The mean of these non-normally distributed values was 5.6 compared to the mean of 9.1 in the C57BL/6 and 0.23 in the CBA distribution. The data in Table II suggest an incomplete dominance situation of anti-NP antibodies at the secondary response, demonstrable by the PFC and antigen-binding methods. This problem is subject to further studies which will be published in a subsequent paper.

Backcrossing into the “recessive” CBA strain produced 87 offspring. The distribution of mean $K_{rel}$ values (sum of D and E in Fig. 4, not presented as such)
was bimodal with a distinct peak coinciding with the CBA peak and a strong shoulder roughly corresponding to the F₁ mice. The distribution suggested that the fine specificity of anti-NP antibodies is controlled by a small number of unlinked loci.

![Graph showing inhibition of NP-phage inactivation by NP aminocap, NIP aminocap, or NNP aminocap.](image)

**Fig. 3.** Inhibition of NP-phage inactivation by NP aminocap, NIP aminocap, or NNP aminocap. The anti-NP antibodies were obtained from individual mice 16 days after secondary injection of NP-CG. Two individual mouse sera from each strain are represented as black or white circles. A hapten concentration was said to have caused 50% inhibition if it increased the number of phage survivors to the survival value of twice-diluted antibody, and 80% inhibition corresponded to the phage survivor count of fivefold-diluted antibody.

**TABLE I**

| Strain                  | No. of mice | Method | Affinity for NP-aminocap (I₅₀)* | Relative affinity‡ for: |
|-------------------------|-------------|--------|--------------------------------|-------------------------|
|                         |             |        | For:                           | NIP-aminocap | NNP-aminocap |
| CBA                     | 9           | PFC    | 22,000                          | 0.66         | 0.52         |
|                         | 40          | HPII   | 570                             | 0.21         | 0.25         |
| C57BL/6                 | 7           | PFC    | 46,000                          | 18           | 12           |
|                         | 27          | HPII   | 180                             | 8.5          | 9.8          |
| (CBA × C57BL/6) F₁      | 6           | PFC    | 37,000                          | 5.3          | 4.5          |
|                         | 48          | HPII   | 260                             | 5.8          | 5.4          |

* I₅₀, log mean of the nanomolar concentration causing 50% inhibition of indirect hemolytic plaques (PFC) or haptenated phage inactivation (HPII). For HPII we used 2-ME to inactivate the IgM fraction.

‡ Kᵣₑₑ (relative affinity) is the log mean of individual Kᵣₑₑ values.
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TABLE II

Strain Characteristics of Anti-NP Antibodies, Secondary Response

| Strain         | No. of mice | Method | Affinity for NP-aminocap (I_{o}) | Relative affinity for: |
|----------------|-------------|--------|----------------------------------|------------------------|
|                |             |        |                                  | NIP-aminocap | NNP-aminocap |
| CBA            | 9           | PFC    | 2,700                            | 0.093       | 0.15        |
|                | 15          | ABC*   | 190                              | 0.021       | 0.067       |
|                | 48          | HPII   | 42                               | 0.038       | 0.071       |
| C57BL/6        | 7           | PFC    | 29,000                           | 4.4         | 10          |
|                | 13          | ABC    | 350                              | 1.5         | 1.2         |
|                | 19          | HPII   | 25                               | 10          | 6.9         |
| (CBA × C57BL/6)F₁ | 6          | PFC    | 18,000                           | 1.8         | 1.6         |
|                | 20          | ABC    | 160                              | 0.26        | 0.41        |
|                | 55          | HPII   | 23                               | 1.5         | 0.91        |

* Inhibition of antigen binding. For other explanations see Table I.

TABLE III

Amount and Affinity of Anti-NP Antibodies in the Secondary Response to NP-BSA

| Strain         | No. of mice | ME-resistant titer (log)* | Affinity for NP-aminocap (I_{o}) | Relative affinity for: |
|----------------|-------------|---------------------------|----------------------------------|------------------------|
|                |             |                           |                                  | NIP-aminocap | NNP-aminocap |
| CBA            | 7           | 3.9 (0.36)‡              | 97                               | 0.25        | 0.19        |
| C57BL/6        | 8           | 4.4 (0.35)‡              | 230                              | 6.0         | 5.2         |

* Amount and affinity of the antibodies were measured using the HPII test. All the tests were performed using 2-ME to inactivate the Ig-M fraction. For explanations see Table I.
‡ Standard error.

More informative distributions were obtained when the animals were divided into two groups, those carrying the C57BL/6 allotype (b positive) mice, and those not carrying it (Ig-1^a^) (Fig. 4 D and E). The distribution of b-negative mice (Fig. 4 E) was indistinguishable from that of CBA mice indicating that an allotype-linked gene is necessary for the C57BL/6 specificity characteristic. The b-positive mice (Fig. 4 D) resembled F₁ animals (Fig. 4 C) more than b-negative backcross animals (Fig. 4 E). There was not a statistically significant difference between the two Ig-1^a^ populations (Fig. 4 C and D). These results indicate that specificity of the anti-NP antibody is controlled by a gene linked to the IgC₇ genes.

Backcrossing to the C57BL/6 strain (Fig. 4 F and G) yielded 33 offspring. Both Ig-1^b^ and Ig-1^a^ animals resembled the C57BL/6 parental strain in their distribution.
Fine Specificity Characteristics in Selected Strains of Mice. At Dr. Potter's suggestion we tested his CB20 congenic inbred strain of mice who have the IgC\textsubscript{H} genes of C57BL/Ka in a BALB/c background genome and the Bailey RI strains that have varying combinations of C57BL/6 and BALB/c genes. To study the role of the H-2-linked genes we also studied three congenic strains that had different H-2 in C57BL/10Sn background.

Specificity of the primary response anti-NP antibodies in these strains was exclusively determined by a gene(s) linked to the IgC\textsubscript{H} locus (Fig. 5). CB20
Fig. 5. Frequency distribution of different mean $K_{rel}$ classes among mice of parental inbred strains and the congenic and recombinant inbred strains of different genotypes. (the HPII method, for explanations see footnote to Fig. 4).
antibody was indistinguishable from anti-NP of the IgC\textsubscript{H}-gene donor and very different from the BALB/c antibody. The Bailey recombinant strains C × BD, C × BE, C × BH, C × BI, and C × BK (Ig-1\textsuperscript{a} strains) had heteroclitic antibody like C57BL/6, while only C × BG and C × BJ (Ig-1\textsuperscript{a} strains) had nonheteroclitic antibody like BALB/c.

Our data do not suggest that H-2-linked genes have a role in determining the specificity of the anti-NP antibodies. Mice with the C57BL/10Sn background were indistinguishable regardless of whether their H-2 was a, k, d, or b (Fig. 5).

Discussion

Data reported in this paper suggest that specificity (cross-reaction) of antihapten antibodies can be used as a Mendelian marker of the V genes. In this study anti-NP antibodies of the primary response were investigated.

The parameter that we chose to quantitate was the mean of two relative affinities, NIP relative to NP and NNP relative to NP. CBA and C57BL/6 mice could be easily distinguished in this way, all 27 C57BL/6 mice having a value higher than 2.0 and all 40 CBA mice a value lower than 1.5 (95% of CBA mice had a value <0.3). We call the C57BL/6 anti-NP antibodies heteroclitic antibodies, and the CBA nonheteroclitic.

Limited data on BALB/c, C57BL/Ka, and C57BL/10Sn anti-NP antibodies are also reported. BALB/c mice resembled CBA while the two other resembled the C57BL/6. These strain characteristics could be demonstrated in IgG antibodies at several times during a 2-3 mo immunization course (14).

The scatter of individual mice in the F\textsubscript{1} generation of CBA × C57BL/6 was wider than the scatter of homozygous mice but it greatly (80%) overlapped with the C57 distribution (Fig. 4). The C57 trait was thus dominant with 80% penetrance.

We produced the backcross generation to the recessive CBA parent and allotyped them. The homozygous a/a mice had a distribution indistinguishable from that of CBA mice while the heterozygotes (Ig-I\textsuperscript{a}) were very similar to \( F_1 \) mice (Fig. 4). This suggested that the observed polymorphism in the specificity of anti-NP antibodies is mainly controlled by one gene locus linked to the H-chain allotype.

The predominant role of the allotype-linked gene(s) in the control of anti-NP specificity was confirmed by antibodies of selected homozygous strains. Congenic mice carrying C57 IgC\textsubscript{H} region in the BALB/c background produced anti-NP of the C57 type. In the seven Bailey recombinant strains anti-NP was heteroclitic or nonheteroclitic depending on the allotype, other genes seemed to have little influence on the specificity (Fig. 5).

While the antibody specificity of inbred strains was approximately the same in the primary and in the secondary response, the rules of inheritance were more complicated in the secondary than in the primary response. The allotype-linked gene(s) is important in the secondary response but one or more allotype-unlinked genes may be involved (Imanishi and Mäkelä, unpublished observations). As our
first studies mainly dealt with the secondary response they led us to suggest multiple-gene inheritance (21).

The fine specificity of the anti-NP antibodies exhibited several characteristics of a V-gene marker: it was allotype linked, it affected specificity rather than quantity of anti-NP, it was independent of the carrier molecule, and it was independent of the H-2 loci. Another similar marker was found in the fine specificity of anti-(5-bromo-4-hydroxy-3-nitrophenyl)acetyl antibodies (Imanishi and Mäkelä, manuscript in preparation).

In rabbits VH genes have allotypes, and could thus be shown to be closely linked to the genes coding for the C regions (22–25). In the mouse five probable V genes have been described: the gene controlling the idiotype found in antiarsonate antibodies of A/J and AL/N mice (7), the gene controlling the idiotype in the antistreptococcal A carbohydrate antibody in A/J mice (8), and the gene controlling the antiphosphorylcholine antibody with the S107 idiotype in BALB/c mice (5). Recently Liebermann et al. (9) could show in BALB/c mice natural antibodies to phosphorylcholine. These antibodies shared idiotypic determinants with the T15 myeloma protein of BALB/c mice. All the five genes were found to be linked to the H-chain allotype. Our experiments are in agreement with a number of observations suggesting a close linkage of V genes and the H-chain C genes.

Fine specificity can probably complement idiotypes as markers of V genes. Both idiotype and fine specificity so far share the disadvantage of being indirect indicators of the genotype. Ontogenic steps between the genotype and the antibody probably include a nearly random choice of one VH and one VL gene in each lymphocyte. One of the genes may be paternal and the other maternal. This is likely to complicate rules of inheritance, and they may be further complicated by antigenic selection of cells which must take place during the immunization. These ontogenic and antigenic selection events probably explain the great differences between genotypically identical F1 mouse individuals. They may also explain why different rules of inheritance were observed for the fine specificity in the primary and in the secondary response. A part of these complications may be avoided in the future as we could demonstrate inherited fine specificity characteristics in the “natural” antibodies of unimmunized mice (Mäkelä and Imanishi, manuscript in preparation).

Dominance of the C57BL/6 allele over the CBA allele was unexpected since the CBA antibody had as high a final affinity for the immunogen as the C57 antibody. An explanation may be that the C57 allele generates a higher number of anti-NP precursor cells than the CBA allele. This is supported by some of our unpublished findings. Young (10-days old) CBA mice failed to respond to NP-CG while C57 mice of this age responded. The other finding was that when CG-primed irradiated (CBA × C57BL/6) F1 mice were reconstituted by $30 \times 10^8$ CBA spleen cells and then immunized by NP-CG they did not respond. Other similarly primed F1 mice reconstituted by $30 \times 10^8$ C57 spleen cells produced anti-NP antibody.

Summary

Our data suggest that fine specificity of antihapten antibodies is a useful
Mendelian marker of variable (V) genes. We found that some mouse strains (e.g., C57/BL6) consistently produced heteroclitic anti-(4-hydroxy-3-nitrophenyl)acetyl (NP) antibodies (relative affinity for related (4-hydroxy-5-iodo-3-nitrophenyl)acetyl and (4-hydroxy-3.5-dinitrophenyl)acetyl was always > 2) while other strains (e.g., CBA) produced “conventional” anti-NP antibodies (relative affinities were consistently < 1).

48 (CBA × C57BL/6)F1 mice were studied and most of them had heteroclitic anti-NP antibodies. They were backcrossed to the recessive CBA parent, and 87 backcross animals were similarly tested. Those heterozygous for the C57BL/6 heavy (H)-chain allotype were similar to the C57BL/6 and the F1 mice while mice homozygous for the CBA allotype were indistinguishable from the CBA. Such monogenic inheritance was observed only in the primary response.

Predominance of allotype-linked genes in the control of the fine specificity characteristics was confirmed by immunizing selected homozygous mouse strains. These mice contained various mixtures of genes from C57BL, BALB/c, and other strains. Specificity of their anti-NP was exclusively determined by genes linked to the H-chain allotype, no influence could be attributed to other genes including the H-2-linked genes.

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