A Genetic Marker in the Variable Region of Rabbit Immunoglobulin Heavy Chain

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A previous study [Mole *et al.* (1971) *Biochem. J.* 124, 301–318] showed several differences in sequence between the variable (V) regions of rabbit immunoglobulin Aa1 and Aa3 heavy chains. The inheritance of one such difference has been followed in a family of 38 rabbits by a radioautographic peptide-'map' technique and is shown to segregate in a Mendelian fashion. This clearly demonstrates the presence of a genetic marker in the rabbit heavy-chain V region, although the finding that Aa2 and Aa3 heavy chains have identity of sequence in the region studied obscures the relationship of this genetic marker to the *a* locus.

In the preceding paper (Pratt & Mole, 1975) it was shown that there were no differences in primary sequence associated with the constant (C) region of the Fd fragment of rabbit IgG* heavy chains of different *a*-locus specificity. It follows that the *a*-locus specificities correlate, either wholly or in part, with the variable (V)-region sequence differences previously described for the different allotypes a1, a2 and a3 (Wilkinson, 1969; Mole *et al.*, 1971; Fleischman, 1971).

To obtain more detailed genetic information from studies on the rabbit *a*-locus allotypes a precise definition of the chemical basis of the serological markers is required. This has proved difficult to achieve, since there are multiple differences in amino acid sequence between V regions of different allotype (Mole *et al.*, 1971). This complexity almost certainly reflects serological heterogeneity of the allotypic antigenic specificities, as first shown by Oudin (1960) and further clarified by the studies carried out by Kindt *et al.* (1973b).

An alternative approach to the correlative one is to establish directly unique amino acid sequences as genetic markers in their own right. This would be feasible if markers could be identified, and their inheritance followed by a peptide-'map' technique. In a previous study (Mole *et al.*, 1971) it was found that there was a completely different sequence between IgG of allotypes a1 and a3 for residues 80–85, and this difference could be followed by studying the Ts4 peptides, which span residues 80–94.

Cysteine-92 was radioactively labelled to allow identification of the peptide by radioautography.

The inheritance of the Ts4 peptide from Aa1 and Aa3 IgG has been studied and the Mendelian pattern characteristic of a typical allelic marker was observed. When peptide 'maps' of Aa2 C-1 fragment were studied the same Ts4 pattern as for Aa3 C-1 fragment was observed; further, sequence analysis showed the major sequences to be identical.

Thus a chemically defined genetic marker has been established in the V region of rabbit IgG. This marker may be different from but closely linked to the serological *a*-locus markers.

Materials and Methods

Rabbits

All the rabbits used in this study originated from crosses between a New Zealand White Aa1 buck (P & H Rabbits, Standlake, Oxon, U.K.) and two Park Farm Grey Aa3 does. The Park Farm Grey (Oxford University Farm, Northmoor, Oxon., U.K.) rabbits are the products of the following mating regime: the does from a blue Beveren X Chinchilla mating were crossed with a New Zealand White male, and the progeny from this mating were then interbred. The line was set up in 1961 and interbreeding has been strictly followed since.

The mating of the Aa1 buck with each of the two Aa3 females gave ten F1 progeny; a further generation was derived from either a series of F1 × parental backcrosses (20 progeny) or an F1 × F1 cross (five progeny). Individual sera were collected from the total of 38 rabbits during age 2–6 months; both the sera and the IgG isolated from them were typed for their '*a*'-locus specificity (or specificities) by using anti-*a* locus antisera prepared and standardized

* Abbreviations: IgG, immunoglobulin G; Aa1, 2 or 3, allotype, *a* locus 1, 2 or 3; Cm-Cys, *S*-carboxymethyl-cysteine; $\square$ Glu, pyrrolid-2-one-5-carboxylic acid; Nap, 4-azido-2-nitrophenol; As, *p*-azophenylarsonic acid; Lac, *p*-azophenyl β -lactoside; solvent PAW, pyridine-pentan-1-ol-water (9:9:7, by vol.), pH 7.0.

as described in the preceding paper (Pratt & Mole, 1975).

Proteins and fragments

IgG was prepared as described by Prahl & Porter (1968), except that, instead of using a gradient from 0.02–0.2M-potassium phosphate buffer, pH 6.3, to elute the IgG from a DEAE-Sephadex column, the column was equilibrated and developed with 0.07M-sodium phosphate buffer, pH 6.3.

Anti-Nap (4-azido-2-nitrophenol) IgG, isolated from a pool of anti-Nap sera from Aa3 homozygous rabbits by the use of the immunoadsorbent Dnp-lysyl-Sepharose 4B (Press *et al.*, 1971), was a gift from Miss E. M. Press.

The following proteins of limited heterogeneity were also studied. The IgG proteins TK 4035 and TK 4135 against streptococcal polysaccharide were raised (Krause, 1970) and isolated as described by Kindt *et al.* (1972); these proteins were purified and supplied by Dr. T. J. Kindt. The IgG proteins 490 As, 490 Lac and 453 Lac were raised to either the *p*-azophenylarsonic acid hapten (As) or the *p*-azophenyl β -lactoside hapten (Lac) as described by Koshland *et al.* (1966), proteins 490 As and 490 Lac coming from the same rabbit; the purified proteins were supplied by Dr. M. E. Koshland. Heavy chains were prepared from IgG as described by Prahl & Porter (1968). As a routine both IgG and heavy-chain preparations were checked for purity by electrophoresis in sodium dodecyl sulphate–polyacrylamide gels by using the method of Fairbanks *et al.* (1971). Heavy chains were cleaved with CNBr in 70% (w/v) formic acid (Givol & Porter, 1965) to yield the *N*-terminal 250 residue fragment, the C-1 fragment.

C-1 fragment was isolated by using a modification of the method of Fruchter *et al.* (1970), which solved the problem of C-1 fragment aggregation. It was observed that the aggregation, which may lead to contamination by Fc fragments, was due to the prolonged dialysis of the CNBr digest against 6M-urea–0.2M-sodium formate buffer, pH 3.3. Therefore after cleavage the heavy-chain fragments (150mg in 7.5ml of solvent) were desalted on a Sephadex G-25 (coarse grade) column (35cm $\times$ 2.8cm) in the above buffer (the salt fraction can be collected under 2M-NaOH to destroy the CNBr). The protein fraction (23–28ml) was applied immediately to a Sephadex G-100 column (140cm $\times$ 3.3cm) in the same urea buffer. The extent of aggregation of C-1 fragment was thus decreased to less than 5% for Aa1 and Aa2 and to less than 10% for Aa3.

Complete reduction and [14 C]alkylation of fragment C-1

Fragment C-1 (3mg) was dissolved in 0.3ml of 7M-guanidine-HCl–0.5M-Tris-HCl, pH 8.2; 13.5 μ l

of dithiothreitol (10mg/ml in water) was added with continuous stirring. After 1h a mixture of 15 μ l of 0.1M-iodoacetic acid and 25 μ Ci (10 μ l) of iodo[14 C]-acetic acid (33 mCi/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) was added and the solution stirred for a further 20min in the dark. The reaction was stopped by the separation of protein from reagents on a Sephadex G-25 (coarse grade) column (1cm $\times$ 30cm) in 0.5M-acetic acid. Fractions of volume 1ml were collected and the protein peak (between 6 and 10ml) was detected by its radioactivity.

Tryptic digestion of C-1 fragment

After being freeze-dried, completely reduced and [14 C]carboxymethylated C-1 fragment was taken up in 0.5ml of 1% (w/v) NH_4HCO_3 , pH 8.2, for tryptic digestion. To the C-1 fragment (3mg) solution 30 μ g of TPCK-trypsin (i.e. trypsin that had been treated with L-1-chloro-4-phenyl-3-tosylamino-butan-2-one) was added followed by incubation at 37°C for 1.5h. The tryptic digest was freeze-dried to remove salt, and the digest was taken up in water to give approx. 100000 d.p.m./2 μ l of digest.

Peptide 'maps'

Peptide 'maps' were made on Eastman Chromagram 6061 silica-gel sheets (20cm $\times$ 20cm; Kodak, Kirkby, Liverpool, U.K.) as described by Sargent & Vadlamudi (1968). Each silica plate was used to compare two digests, which were loaded (1–3 μ l in 0.1 μ l portions) at positions 9cm and 11cm along a vertical axis bisecting the plate.

The two origins were then covered with a small polyethylene strip (4cm $\times$ 1cm) and the silica plate was sprayed with pyridine–acetic acid–water (25:1:474, by vol.), pH 6.5. After the spraying, the polyethylene strip was removed and the dry area carefully wetted with pH 6.5 buffer by using a fine capillary. Electrophoresis was carried out along the horizontal axis of the plate at 400V and 8–12mA for 3h in a Shandon SAB-2880 electrophoresis apparatus (Shandon Scientific Co., London N.W. 10, U.K.), after which the plate was allowed to dry in air at room temperature.

When dry, the plate was cut in two along the central horizontal axis, and the two halves were subjected to ascending chromatography in the solvent pyridine–pentan-1-ol–water (9:9:7, by vol.) adjusted to pH 7.0 with acetic acid. From each plate therefore the pair of tryptic digestions would on development assume a 'butterfly-wing' (or mirror-image) pattern on comparison with another (see the Results section).

The peptide 'maps' were then submitted to radioautography. Except for the tryptic digests of the radioactive homogeneous C-1 fragments, each electrophoretogram compared a parental fragment digest with that of one of its progeny.

Radioautography with Kodak BB-54 X-ray film was carried out for 5–6 days, each digest having 90000–120000 d.p.m. As the size of the film used is 53 cm × 35 cm it was possible to compare the 'maps' (20 cm × 10 cm) of each parent with two of their progeny on one film. The four half-plates were fastened to a sheet of chromatography paper, double-sided Scotch tape being used to stick the peptide 'maps' to the paper via their polyethylene terephthalate support, which in turn was taped firmly against the X-ray film.

Other analytical methods, such as peptide isolation, detection and amino acid analysis, were carried out as described in the preceding paper (Pratt & Mole, 1975). Large tryptic peptides, containing only one of the four heavily ^{14}C -labelled *S*-carboxymethyl-cysteine residues of fragment C-1, were isolated from a digest of citraconylated C-1 fragment of either Aa1 or Aa3 specificity as described by Mole *et al.* (1971).

Ts4 peptides of Aa1 and Aa3 IgG

As shown in Fig. 1, most of the known differences in sequence between Aa1 and Aa3 heavy-chain V regions occur in the N-terminal 33 residues and in positions 80-85 (Wilkinson, 1969; Mole *et al.*, 1971). The six consecutive differences in the region 80-85 form the most striking difference between the two allotypes. This region is included in the Ts4 peptides

Further studies on the Ts4 peptides of the two allotypes revealed that the Aa1 Ts4 peptide had an electrophoretic mobility of -0.37 at pH 6.5 [relative to aspartic acid (-1) and ϵ -Dnp-lysine (0)], which would indicate (Offord, 1966) that residue 85 was in fact a glutamic acid residue, and not glutamine as assigned by O'Donnell *et al.* (1970). However, as 50% formic acid had been used as solvent in the isolation of the Aa1 Ts4 peptide (Mole *et al.*, 1971), it was thought possible that deamination of glutamine-85 could have occurred, which would explain this discrepancy.

Re-isolation of the Aa1 Ts4 peptide from C-1 fragment by using the conditions of O'Donnell *et al.* (1970) did, however, confirm that the mobility of this peptide at pH6.5 was -0.37 , and residue 85 is therefore glutamic acid and not glutamine. Thus the Aa1 Ts4 peptide had an electrophoretic mobility at pH6.5 of -0.37 , whereas the major-yield Aa3 Ts4 peptide had a mobility of -0.21 [relative to aspartic acid (-1) and ϵ -Dnp-lysine (0)].

One further problem still had to be clarified, however, concerning a minor-yield Aa3 Ts4 peptide variant. As stated by Mole *et al.* (1971), the Aa3 Ts4 peptide was isolated as two electrophoretically distinct forms, A and B, the former having the same mobility at pH6.5 as the Aa1 Ts4 peptide. Because of the probability that peptide Aa3 Ts4-A would interfere in this study, it was further characterized.

Aa1	Pool	[Glu-Ser- ^{Val} _{Leu} -Glu-Glu-Ser-Gly-Gly-Arg-Leu-Val-Thr-Pro-Thr-Pro-Gly-Leu-	10
Aa3	Pool	[Glu-Ser-Leu-Glu-Glu-Ser-Gly-Gly- ^{Asp} _{Val} -Leu-Val-Lys-Pro-Gly-Ala-Ser-Leu-	
Aa1	Pool	Thr-Leu-Thr-Cys-Thr- ^{Val} _{Ala} -Ser-Gly-Phe-Ser-Leu-Ser-Ser-Tyr- ^{Ala} _{Asp} -Met	20 30
Aa3	Pool	Thr-Leu-Thr-Cys-Thr-Ala-Ser-Gly-Phe-Asn- ^{Gly} _{Ala} -Ser-Ser- ^{Tyr} _{Phe} -Tyr-Met	
Aa1	Pool	Met- ^{Ile} _{Lys} -Thr-Ser-Pro-Thr-Glu-Asp-Thr-Ala-Thr-Tyr-Phe-Cys-Ala-Arg	80 85 90
Aa3	Pool	Met- ^() -Thr-Ser-Leu-Thr-Ala-Ala-Asp-Thr-Ala-Thr-Tyr-Phe-Cys-Ala-Arg	

Fig. 1. Amino acid sequence of residues 1–34 and 79–94 for allotypes a1 and a3

The sequence was derived from results obtained with pooled normal IgG (Wilkinson, 1969; Mole *et al.*, 1971).

Peptide Aa3 Ts4-A had been isolated in a yield of 10–20% of that of peptide Aa3 Ts4-B, and was shown by Mole *et al.* (1971) to have an amino acid composition identical with that of the latter peptide. Re-isolation of the two peptides confirmed this; however, whereas sequence analysis gave the expected *N*-terminal sequence, Thr-Ser-Leu, for peptide Aa3-Ts4-B, the Aa3-Ts4-A peptide gave nothing at each step. It was concluded from this result that peptide Aa3-Ts4-A was an *N*-formylated form of peptide Aa3-Ts4-B, i.e. an artifact from the use of 50% formic acid in a purification step. This conclusion was given support by the fact that, when 0.05M-NH₃ was used as fractionating solvent, no Aa3-Ts4-A peptide was detectable. Also, if the peptide was *N*-formylated the *N*-terminal amino acid would be regenerated on acid hydrolysis and thus yield a composition identical with that of the B form, as was found to be the case.

With these problems clarified, it seemed probable that a radioautographic peptide-‘map’ technique could be used to distinguish between Aa1 and Aa3 C-1 fragments, on the basis of their Ts4 peptides, after the fragments were [¹⁴C]alkylated with iodo-[¹⁴C]acetic acid (to label the Ts4-peptide cysteine residue) and digested with trypsin.

Yield of Aa1 and Aa3 Ts4 peptides

After radioalkylation of cysteine-92 the yield of the Ts4 peptides from tryptic digests of the C-1 fragment of both allotypes could be estimated (Mole *et al.*, 1971) by making use of the specific activity of the radioactive chymotryptic peptide CmCys-Ala-Arg (residues 92–94), which is common to both allotypes. It was found that tryptic digestion of citraconylated Aa1 or Aa3 C-1 fragment released the Ts4 peptides in yields of 20 and 50% respectively as a consequence of the presence of a fractional methionine at position 79.

In both allotypes the rest of the CmCys-Ala-Arg marker peptide was derived from a peptide (C-Ts4) about 30 residues long. Tryptic digestion of the C-Ts4 peptides after removal of the citraconyl groups, released Ts4 peptide in greater than 95% yield from the Aa1 peptide (as a consequence of a lysine residue at position 79), but no Ts4 peptide was released from Aa3 C-Ts4 peptide. The final yield of the Ts4 peptides on tryptic digestion of Aa1 and Aa3 C-1 fragments should therefore be about 95 and 50% respectively. Aa3 peptide C-Ts4 was found close to the origin on electrophoresis at pH 6.5 and therefore does not interfere in the peptide ‘maps’.

Peptide ‘maps’

After considerable experimentation peptide ‘maps’ run as described in the Materials and Methods section

gave a satisfactory resolution of peptides. The chromatography step presented particular problems, and both butan-1-ol-acetic acid-water-pyridine [15:3:2:10 (by vol.) (Waley & Watson, 1953)] and butan-1-ol-acetic acid-water [12:3:5 (by vol.) (Press *et al.*, 1966)] solvents gave poor results with the silica support used. A successful solvent was obtained when a modification of the pyridine-3-methylbutan-1-ol-water (7:7:6, by vol.) mixture described by Edman (1946) was used. The volume proportions of the constituent solvents were changed, pentan-1-ol was substituted for 3-methylbutan-1-ol and the pH was lowered from 9.0 to 7.0 with a few drops of acetic acid. The final mixture selected was pyridine-pentan-1-ol-water (9:9:7, by vol.), pH 7.0 (solvent PAW).

The electrophoretic dimension proved troublesome, because separation of the peptide Ts3 from the Aa1 Ts4 peptide (see Plate 1) was dependent not simply on the pH but also on the concentration of the pyridine-acetate buffer used. Pyridine-acetic acid-water (25:1:474, by vol.) gave separation, whereas stronger buffers did not. Also, this buffer could only be used effectively if kept for 2–3 days under 1–2 litres of air; the reason for the effect of storage is unknown, as no change in pH was detectable.

The poor solubility of Aa3 peptide Ts4 also presented some problems. For successful results a maximum of 50 µg of digest was applied to the plate, and also, care was taken to ensure that the plate was not dried too thoroughly on application of the sample or before chromatography. If these points were observed and the techniques carried out exactly as described in the Materials and Methods section, well-resolved peptide ‘maps’ were reproducibly obtained.

The separation obtained is demonstrated in Plate 1. Rabbit M186 has the a1 serotype, whereas rabbit 681M has the a3 serotype. The radioactive peptide spot Ts3 spans residues 124–149 of the C region of fragment C-1 and is therefore identical for both allotypes (Pratt & Mole, 1975). Being common to both allotypes and being proximal to both the Aa1 and Aa3 Ts4 peptides, peptide Ts3 is used as an internal standard in interpretation of the ‘map’. Peptide Aa1 Ts4 (designated Ts4-1) moves anodic to peptide Ts3 and has a slightly lower *R_F* value on chromatography, whereas the Aa3 Ts4 peptide (designated Ts4-3) moves cathodic to peptide Ts3 but has a higher *R_F* value in the second dimension.

Identification of radioactive tryptic peptides

As it was apparent from the peptide ‘maps’ (see Plate 1) that the differences between the radioactive peptides of Aa1 and Aa3 were not confined to the Ts4 peptides, an attempt was made to identify the source of the various peptide spots. Large tryptic

EXPLANATION OF PLATE I

Cross of Ts4-1-peptide homozygote and Ts4-3-peptide homozygote

The Plate shows radioautographs of tryptic digests of C-1 fragments of Ts4-3-peptide homozygote parent (681M), Ts4-1-peptide homozygote parent (M186) and progeny HET X and HET G, showing the presence of both Ts4 peptide forms in the offspring. The directions of electrophoresis (pH 6.5) and chromatography (solvent PAW) are indicated; for details of solvent systems see the text.

EXPLANATION OF PLATE 2

Breakdown of Aa1 tryptic peptide 'map' into its component peptides

The Plate shows radioautographs of tryptic digests of Aa1 C-1 fragment and the large purified tryptic peptides. The C-region fragments T2 and T3 generate peptides Ts2 and Ts3. From the V region are generated a cluster of peptides containing cysteine-22 (Aa1 C-1-4) and the peptides Ts4-1a and Ts4-1b, containing cysteine-92, the difference of which is linked to the presence of lysine (C-Ts4-derived) or methionine (Ts4-derived) at position 79. The directions of electrophoresis (pH 6.5) and chromatography (solvent PAW) are indicated; for details of solvent systems see the text.

EXPLANATION OF PLATE 3

Backcross of Ts4-1-peptide homozygote with Ts4-1-Ts4-3-peptide heterozygote

The Plate shows radioautographs of tryptic digests of C-1 fragments of Ts4-1-peptide homozygote (M186), Ts4-1-Ts4-3-peptide heterozygote (HET E) and progeny E1 and E2 showing progeny only capable of giving parental phenotype. The directions of electrophoresis (pH 6.5) and chromatography (solvent PAW) are indicated; for details of solvent systems see the text.

EXPLANATION OF PLATE 4

Similarity of Aa2 and Aa3 tryptic peptide 'maps'

The Plate shows a radioautograph of tryptic digest of normal Aa2 C-1 fragment showing similarity to that of normal Aa3 C-1 fragment digest (see Plate 5). The homogeneous anti-streptococcal antibody C-1 fragment digests, TK 4035 (Aa2) and TK 4135 (Aa3), similarly resemble one another and those obtained in the pooled situation. The directions of electrophoresis (pH 6.5) and chromatography (solvent PAW) are indicated; for details of solvent systems see the text.

EXPLANATION OF PLATE 5

Comparison of normal Aa3 digest with Aa3 anti-Nap digest

The tryptic peptide 'maps' of purified anti-Nap C-1 fragment (Aa3) and normal Aa3 (681M) are compared: no difference is apparent. Also shown is a comparison of the digests of normal Aa1 C-1 fragment (M186) and that of a homogeneous anti-Lac antibody (453 Lac) derived from an Aa1 animal. The latter digest shows no Ts4-1 peptide but a peptide in the Ts4-3 position; for explanation see the text. The directions of electrophoresis (pH 6.5) and chromatography (solvent PAW) are indicated; for details of solvent systems see the text.

peptides containing only one of the cysteine residues of the four intrachain disulphide bridges of the C-1 fragment had previously been purified from a tryptic digest of citraconylated C-1 fragment for both allotypes (Mole *et al.*, 1971; Pratt & Mole, 1975). Therefore, after the unblocking, peptide 'maps' were produced from tryptic digests of these large tryptic fragments.

Peptide 'maps' of the tryptic digests of the large C-region tryptic peptides T2 (residues 177–253) and T3 (residues 95–170) of either allotype were identical; this was an expected result, since the C regions of both allotypes has been shown to have identical primary sequences (Pratt & Mole, 1975). The tryptic digest of both Aa1 and Aa3 peptides T3 gave only one radioactive peptide spot, peptide Ts3 (described in Plate 1). The major radioactive peptide (peptide Ts2) of the peptide T2 digest (Plate 2, top right) remained at the origin in both the electrophoretic and chromatography dimensions. This insolubility was, however, due to the high protein load needed because of the low specific radioactivity of the T2 peptide isolated by the procedure of Mole *et al.* (1971). The C-1 fragment digests used in the peptide inheritance study were, however, of much greater specific radioactivity, and therefore less peptide material was applied at the origin. Under these conditions the Ts2 peptide (Plate 2, top left) moved towards the cathode on electrophoresis but had R_F 0 in the chromatography phase.

Comparison of the tryptic digests of Aa1 and Aa3 N-terminal 33-residue (C-1-4) peptides showed further allotype-related peptide spots. The Aa1 C-1-4 digest (Plate 2, bottom left) gave a cluster of neutral or slightly acidic peptides of high R_F in the chromatography phase. The Aa3 C-1-4 fragment digest (not shown) had no peptides in this region (compare M186 and 681M in Plate 1). The major peptides were more acidic and, with the 3 h electrophoresis time used, some Aa3 C-1-4 peptides 'ran off' the plate. Under the electrophoresis conditions used it was therefore not possible to differentiate between an a^1a^1 homozygote and an a^1a^3 heterozygote by virtue of the radioactive peptides from this region; however, a shorter electrophoresis time may show peptides unique for the Aa3 C-1-4 fragment.

As previously described (Mole *et al.*, 1971), the Ts4 region for both allotypes is present in two column fractions after tryptic digestion of citraconylated C-1 fragment. One fraction contains the Ts4 peptides themselves (released by cleavage of the fractional methionine at position 79). The larger C-Ts4 fraction (residues 67–94) contains the Aa1 or Aa3 Ts4 region from those molecules not having this fractional methionine.

The radioactive peptide from the Aa3 C-Ts4 tryptic digest did not move significantly from the

origin in either 'map' dimension; however, the Aa1 C-Ts4 peptide, because of the methionine/lysine replacement at position 79 (Mole *et al.*, 1971), quantitatively released the Aa1 Ts4 peptide (peptide Ts4-1a; top right of Plate 2).

Of the Ts4 peptide column fractions, the Aa3 Ts4 fraction gave a single radioactive spot running in a position identical with that marked Ts4-3 in Plate 1: the Aa1 Ts4 fraction, however, gave two radioactive spots. One peptide spot (Ts4-1a; Plate 2, bottom right) was shown to have a composition identical with that of the Ts4 peptide derived from tryptic digestion of the Aa1 C-Ts4 peptide (see Fig. 1), whereas the Ts4-1b peptide lacked isoleucine and proline but had an increased leucine and threonine content. It would seem that these variants are linked to the presence or absence of methionine at position 79. Thus the Ts4-1b peptide is released from molecules having methionine at position 79 (and hence is present in the Ts4 fraction) and the Ts4-1a variant from those molecules with lysine at position 79 (C-Ts4 fraction). The small amount of Ts4-1a peptide in the Aa1 Ts4 fraction is presumably due to incomplete citraconylation.

This finding explains the presence of a double spot in the Aa1 Ts4 peptide position of the rabbit M186 'map' (Plate 2, top left). That the Ts4-1a peptide spot is the more intense reflects the predominance of lysine (80% or greater) in Aa1 molecules at position 79.

Inheritance of the Ts4 peptide

The various matings used in this study, the *a*-locus serotype of the parents and their offspring and the presence or absence of the Ts4-1 and Ts4-3 marker peptides in the subsequent peptide 'maps' are summarized in Table 1. As expected, the crosses between the Ts4-1 homozygous male M186 and the two Ts4-3 homozygous females 381D and 681M resulted in progeny (HET A–HET G and HET W–HET Y) having both Ts4 marker peptides, as is evident in Plate 1. 'Maps' identical with those of progeny HET G and HET X were obtained for the other F1 generation rabbits.

The backcrosses between rabbit M186 and the F1 rabbits HET E and HET F produced 13 progeny, six of which (rabbits E2, E6 and F1–F4) were peptide-Ts4-1 homozygotes, the other progeny bearing IgG from which both peptides Ts4-1 and Ts4-3 could be seen. Plate 3 shows the 'maps' of the E1 and E2 progeny of the M186 × HET E backcross. Rabbit E1 possesses IgG from which both the Ts4 peptides could be seen whereas rabbit E2 was 'typed' as a Ts4-1-peptide homozygote. No rabbit from these crosses that possessed IgG from which only the Ts4-3 peptide could be obtained was revealed. In the complementary backcross, HET A × 681M, two of the

Table 1. *Inheritance of Ts4-1 and Ts4-3 peptides: a summary of the breeding study, listing origin of parents and progeny*

+ or - indicate the presence or the absence in serum of *a*-locus determinants determined by using specific antisera (*a*-locus serotype) and the presence or the absence in C-1 fragment tryptic digests of peptides Ts4-1 and Ts4-3 (Ts4 peptide).

		<i>a</i> -locus serotype		Ts4 peptide	
		1	3	1	3
Sire	M186	+	-	+	-
Dam	381D	-	+	-	+
Progeny	HET A	+	+	+	+
	B	+	+	+	+
	C	+	+	+	+
	D	+	+	+	+
	E	+	+	+	+
	F	+	+	+	+
	G	+	+	+	+
Sire	M186	+	-	+	-
Dam	681M	-	+	-	+
Progeny	HET W	+	+	+	+
	X	+	+	+	+
	Y	+	+	+	+
Backcrosses					
Sire	M186	+	-	+	-
Dam	HET E	+	+	+	+
Progeny	E1	+	+	+	+
	E2	+	-	+	-
	E3	+	+	+	+
	E4	+	+	+	+
	E5	+	+	+	+
	E6	+	-	+	-
	E7	+	+	+	+
	E8	+	+	+	+
Sire	M186	+	-	+	-
Dam	HET F	+	+	+	+
Progeny	F1	+	-	+	-
	F2	+	-	+	-
	F3	+	-	+	-
	F4	+	-	+	-
	F5	+	+	+	+
Sire	HET A	+	+	+	+
Dam	681M	-	+	-	+
Progeny	AM1	-	+	-	+
	AM2	+	+	+	+
	AM3	+	+	+	+
	AM4	+	+	+	+
	AM5	+	+	+	+
	AM6	-	+	-	+
	AM7	+	+	+	+
F1 × F1 Cross					
Sire	HET D	+	+	+	+
Dam	HET C	+	+	+	+
Progeny	DC1	+	+	+	+
	DC2	+	-	+	-
	DC3	+	-	+	-
	DC4	+	+	+	+
	DC5	+	+	+	+

progeny (AM1 and AM6) were typed as Ts4-3-peptide homozygotes, the other rabbits having IgG in which both Ts4 peptides could be seen. Again no peptide-Ts4-1 homozygote rabbits were evident.

Although the number of progeny from a given type of cross was not large, a total of 38 rabbits within the same family were studied for the inheritance of the defined Ts4 peptides. In all cases the Ts4-peptide variants were inherited in a simple Mendelian fashion, indicative of genetic markers. Although in each case the actual serotype of the rabbit under study was exactly that expected from the chemical typing (see Table 1), it cannot be stated that the Ts4 peptides form part of their respective *a*-locus specificities, as they are serologically inactive. It is clear, however, that the Ts4 peptides represent V-region genetic markers closely linked to, but not necessarily part of, the serological *a*-locus specificities.

Radioautography of Aa2 fragment C-1

With a view to making the method applicable to the differentiation of all three *a*-locus allotypes, radioautographs of a tryptic digest of Aa2 C-1 fragment were prepared. The Aa2 C-1 fragment was isolated from the pooled IgG from five homozygous Aa2 New Zealand white rabbits.

Comparison of the Aa2 C-1 fragment radioautograph with those of Aa1 (Plate 4) and Aa3 (681M; Plate 5) C-1 fragment 'maps' showed that, although it was possible to distinguish between the a1 and a2 allotypes, no difference was apparent between the Aa2 and Aa3 tryptic peptide 'maps'. The Aa2 'map' showed the presence of a radioactive peptide moving in the same position as the Aa3 Ts4 peptide. Electrophoresis at pH4.5, 5.5 and 8.9 failed to differentiate between the Aa2 and Aa3 C-1 fragments; similarly, no difference was apparent between the two digests with the use of a variety of solvents in the chromatography dimension, suggesting that the two Ts4 peptides were very similar.

The Aa2 Ts4 peptide was therefore isolated from a tryptic digest of citraconylated Aa2 fragment C-1. As for allotype Aa3, described above, two radioactive peptides were isolated from the Aa2 Ts4 fraction, peptides Aa2-Ts4-A and Aa2-Ts4-B: peptide Aa2-Ts4-A had a mobility at pH6.5 of -0.35 and gave no *N*-terminal residue on dansylation (presumably owing to *N*-formylation), whereas peptide Aa2-Ts4-B had a mobility at pH6.5 of -0.22. Both Aa2 Ts4 peptides had identical amino acid compositions, and moreover their composition was extremely similar to that found for the Aa3 Ts4 peptide (A. N. Barclay & L. E. Mole, unpublished work).

Subjection of the Aa2-Ts4-B peptide to the 'dansyl-Edman' procedure showed the major sequence of the peptide to be identical with that found for the Aa3 Ts4 peptide (Fig. 1), although positions

1 and 2 of the Aa2 Ts4 peptide, predominantly threonine and serine respectively, appeared to be relatively heterogeneous compared with the Aa3 Ts4 peptide. The main finding was, however, that the major sequences of Aa2 and Aa3 Ts4 peptides were the same.

To ensure that the Aa2 IgG used was genuinely of that allotype, the IgG was retested for *a*-locus determinants by using antisera that had been concentrated by Na₂SO₄ precipitation of the original *a*-locus antisera. These concentrated reagents reaffirmed that the IgG used possessed only Aa2 determinants. Moreover, the Aa2 *N*-terminal 34-residue fragment has been isolated and shown to have a sequence very similar to that given by Fleichman (1971) for an Aa2 antibody of limited heterogeneity (A. N. Barclay and L. E. Mole, unpublished work).

The inability to differentiate between 'maps' of Aa2 and Aa3 C-1 fragments was therefore due to their Ts4 peptides being identical in major sequence. Both the Aa2 and Aa3 Ts4 peptides are released on CNBr cleavage at methionine-79; whether the regions 80-94 are identical in molecules of Aa2 and Aa3 C-1 fragments lacking a methionine at position 79 is unknown.

Peptide 'maps' of purified antibodies

Strosberg *et al.* (1972) found that the sequence in the region 80-85 of an Aa1 anti-pneumococcal antibody heavy chain of limited heterogeneity was quite different from the major Aa1 sequence shown in Fig. 1. This result raised some doubt about the interpretation of the studies on pooled normal rabbit heavy chain; some purified antibody preparations were therefore investigated to determine whether with these proteins the Ts4 peptide still correlated with the serological typing.

Six antibody preparations were studied: anti-Nap antibody of allotype a3 from Miss E. M. Press (Press *et al.*, 1971); two anti-streptococcal antibodies of allotypes a2 (TK 4035) and a3 (TK 4135) from Dr. T. J. Kindt; one anti-arsonate antibody (490As) of allotype a1; two Aa1 anti-lactoside antibodies (490 Lac and 453 Lac) from Dr. M. E. Koshland. The 490 As and 490 Lac antibodies were from the same rabbit (Koshland *et al.*, 1966). All antibodies were of limited heterogeneity except for the heterogeneous anti-Nap antibody preparation, which was, however, purified by affinity chromatography and specifically bound 2 mol of Nap-lysine/mol (Press *et al.*, 1971).

The anti-Nap antibody was raised in the same breed of rabbit used in the inheritance study and was a pool of sera from several rabbits. The radioautograph of the anti-Nap C-1 fragment is compared with the 'maps' of Aa1 (M186) and Aa3 (681M) C-1 fragment digests in Plate 5, where the presence of

the Ts4-3 peptide and the complete absence of the Ts4-1 peptide is apparent. The Ts4 peptide from this antibody preparation has been isolated, and the amino acid composition and major *N*-terminal sequence are indistinguishable from those of peptide Ts4-1 from non-immune Aa3 C-1 fragment.

The anti-streptococcal antibodies TK4035 (Aa2) and TK 4135 (Aa3) gave the radioautographs shown in Plate 4. Both 'maps' show a radioactive peptide in the Ts4 peptide position expected for a normal C-1 digest of allotype a2 or a3.

Of the three Aa1 antibodies of restricted heterogeneity, both 490 Lac and 490 As (not shown) gave typical Aa1 fragment C-1 'maps'; however, the 'map' for protein 453 Lac (Plate 5) was clearly more similar to the Aa3 C-1 fragment 'maps', 681M and Aa3 Nap, there being no trace of the Ts4-1 peptide present. However, it is also apparent in the 453 Lac 'map' that the Aa1 C-1-4 radioactive peptide cluster (see Plate 2), which is a consistent feature of Aa1 C-1 fragment 'maps', is also absent. Protein 453 Lac therefore has none of the 'map' characteristics described above for the a1 specificity. Although the protein was derived from a homogeneous Aa1 rabbit, on checking of the *a*-locus allotype against concentrated antisera to the a1, a2 and a3 specificities no precipitin line was obtained, although a precipitin line did develop against horse anti-(rabbit Fc) antisera. It seems probable that protein 453 Lac is a so-called 'blank' molecule, as such molecules have V regions coded for by loci different from the *a* locus (Kim & Dray, 1972) and are also known to have an *N*-terminal 34-residue fragment quite different in composition from the corresponding *a*-locus-derived fragment (Prahll *et al.*, 1973). Further, there are previous reports of homogeneous antibodies raised in rabbits that, on purification, lacked the group-*a* markers (Kindt *et al.*, 1973a). In conclusion, the studies on well-defined antibody proteins confirmed the correlation between Ts4 peptide type and *a*-locus serotype described in the earlier sections of this study.

Discussion

The present study is similar in concept to that conducted by Edelman & Gottlieb (1970), who also detected a difference in primary sequence adjacent to a modifiable half-cystine residue by radioautography of peptide 'maps'. This approach is convenient in that, by detecting only the radioactive peptides, the subsequent peptide 'maps' are simple to interpret, and the difference being followed is little interfered with, an important point in studying heterogeneous immunoglobulins. The conventional peptide-'map' technique used by Edelman & Gottlieb (1970) has been replaced in the present study with the micro peptide-'map' technique described by

Sargent & Vadlamudi (1968), as well as involving a scaling down of methodology at all prior steps. In this way very small amounts of protein could be used in obtaining a radioautograph in only 5 days; moreover, four different proteins could be compared on the same film. Provided that a modifiable residue is available, this technique is therefore thought to be very useful for quick comparison of many samples, especially if only small amounts of material are available.

This technique was used to follow the inheritance of a defined sequence difference in the V region of rabbit γ chains of different a -locus allotype in an effort to establish this difference as a V-region genetic marker. Although it is known that no primary sequence differences are present on comparison of a1 and a3 Fd fragment C regions (Pratt & Mole, 1975), that the isolated V-region domains from a1 and a3 γ chains have all the a -locus specificities (Koshland & Mole, 1975) and indeed that sequence correlates between a1 and a3 V regions are consistently found (Wilkinson, 1969; Mole *et al.*, 1971; Jaton *et al.*, 1973), none of these previous studies has established a defined difference as part of the a -locus specificities or as an heritable genetic marker.

The sequence difference studied in the present work was the glutamic acid/alanine replacement between allotypes a1 and a3 at position 85 of the heavy-chain V region. As this difference involved a difference in peptide charge, it was easily followed with the peptide-'map' technique. The breeding study, involving a total of 38 rabbits, showed the Ts4 peptide sequence difference to be inherited in the simple Mendelian fashion expected for a genetic marker. Although the Ts4 peptide (or peptides) was not purified for each of the rabbits, it seems evident that the defined sequence difference was maintained, because, if glutamic acid-85 was not present in the Ts4-1 peptide, a compensatory mutation would have to have occurred to preserve its mobility at pH 6.5, an unlikely event. Whether alanine-85 in peptide Ts4-3 was maintained is more difficult to judge. It is known, however, to be present in normal pooled heavy chain as well as in anti-Nap heavy chain, and, further, all the homogeneous Aa3 antibodies studied to date have alanine at position 85. It is concluded therefore that the alanine/glutamic acid interchange at position 85 is a *bona fide* V-region genetic marker.

Although this finding argues strongly for a minimum of V-region genes, the finding of at least two Ts4-1 peptide variants indicates that there is more than one V-region gene in animals bearing the Ts4-1 marker. In such animals the composition of the Ts4-1 peptide, and hence presumably its sequence, differs when methionine rather than lysine is present at position 79 (Mole *et al.*, 1971; Jaton *et al.*, 1973).

The presence of both Ts4-1 peptides in animals also bearing the Ts4-3 peptide marker (though this is not clearly visible in the Plates as shown) shows the two Ts4-1 peptide forms to be derived from different structural genes rather than that the two forms are alleles of each other (in which case only one form would be present in the heterozygote). The electrophoretic mobility and composition of the two Ts4-1 forms indicates that the glutamic acid marker is present at position 85 in both cases.

Whether this genetic marker is a part of the serological a -locus determinants is obscured by the finding that the Aa2 and Aa3 Ts4 peptides from pooled heavy chain were identical in composition and major amino acid sequence. This type of variance, i.e. identity of sequence between two allotypes at a given position whereas the third allotype varies, is, however, a feature of some of the sequence correlates in the N-terminal 30 residues. Thus, whereas the a1 and a3 specificities have the residues glutamic acid, glycine and valine at positions 4, 7 and 11 (Wilkinson, 1969; Jaton *et al.*, 1973), allotype a2, whether derived from a pool (A. N. Barclay & L. E. Mole, unpublished work) or a specific antibody (Fleischman, 1971; Jaton & Haimovich, 1974), has the alternative residues lysine, glutamic acid and phenylalanine. Although positions do occur that have different amino acid correlates for all three allotypes, on balance the former pattern of variance between the correlates is in the majority. Todd (1972) has attempted to explain these two types of correlates in terms of the serological allotypic sites. Whether the V-region marker studied here is a part of the a -locus specificities or not, it is obvious from the breeding study that the chemical marker and the serological markers are closely linked.

The evolution of the complexity of these structural correlates is still unclear. That they represent allelic forms (of a single gene) equivalent to the genes for the three human V-region subgroups is not borne out by sequence comparison. Indeed, over the first 30 residues, a stretch rich in subgroup specific residues for other species, the sequences of all three allotypes resemble the human V_{HII} prototype sequence more than those of the V_{HI} and V_{HIH} human subgroups. This situation may be clarified when detailed structural studies of the rabbit V_H x and y loci (Kim & Dray, 1972) are carried out.

Another explanation for the existence of such complex loci is that the actual genetic polymorphism is for the control of gene expression and that each rabbit has all three a -locus genes present (see, for example, Bodmer, 1973). The anomalous expression of Gm allotypes in human mixed lymphocyte cultures (Rivat *et al.*, 1973) lends some support to this view. Strosberg *et al.* (1974) have reported a rabbit of genotype a^1a^3/b^4b^5 expressing the phenotype

Aa1,2,3/b4,5,6 on hyperimmunization with *Micrococcus lysodeikticus*, suggesting a breakdown in regulatory gene function. However, whether the *a*-locus allotypes represent true allelic genes or not, the conclusion from the present study is that the V-region genetic marker identified could be maintained in only a small number of V-region genes.

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