Comparative Peptide Mapping of Radioiodinated Light Chains Derived from Homogeneous Rabbit Anti-Streptococcal Antibodies†

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ABSTRACT: Fingerprints of tryptic peptides containing tyrosine were prepared from 14 anti-streptococcal antibody light chains excised from polyacrylamide gels and labeled with ^{125}I. Comparison of the peptide maps of light chains from anti-group B and anti-group C streptococcal antibodies identified a tyrosine bearing peptide apparently linked to the antigen binding specificity of L chains derived from anti-group C antibodies. No correlation of peptide differences with b allotype was observed. Light chains obtained from antibody raised in an Australian wild rabbit differed by one tryptic peptide from the maps obtained for L chains from laboratory rabbits. Two L chains, derived from antibodies of the same specificity, but produced in separate rabbits, possessed identical ^{125}I labeled tryptic peptide maps. The consequences of this finding are discussed in terms of current theories for the generation of antibody diversity.

The primary structure of the variable regions of antibody light (L) and heavy (H) chains determines the antigen binding specificity of the molecule (Edelman and Gall, 1969; Porter, 1973). Comparison of the V regions from different antibody molecules of the same antigen binding specificity is an obvious means of identifying those residues that determine antibody specificity. The amino acid sequences of the V regions of several L chains from anti-*p*-azobenzoate, anti-streptococcal, and anti-pneumococcal antibodies have been reported (Apella et al., 1973; Chen et al., 1974; Jaton, 1974; Margolies et al., 1975) and the complete V domains of two homogeneous antibodies to type III pneumococcal polysaccharide have recently been sequenced (Jaton, 1975). However, insufficient data exist to enable complete identification of the regions of the V domain that directly relate to antibody complementarity.

This report presents the results of a comparative study of the tryptic peptide maps of radioiodinated rabbit L chains derived from antibodies to either group C or group B streptococcal carbohydrates. Two-dimensional peptide mapping has proved to be an extremely sensitive means for ascertaining primary structure differences in related polypeptide chains (Ingram, 1957) and is capable of detecting one amino acid interchange in comparison of hemoglobin β chains of length 146 residues (Ingram, 1963). We chose this method, coupled with the sensitivity gained by radioiodination of tyrosyl residues of L chains isolated by disc electrophoresis (Bray and Brownlee, 1973), because an overall structural comparison was required and the amount of purified L chains available was insufficient for extensive sequence analysis by any of the methods presently in use. Interpretation of the maps was facilitated by available amino acid sequence data for rabbit L chains. The techniques employed allowed rapid and reliable analysis of the tyrosine containing peptides of small amounts of L chains from partially purified antibodies.

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Materials and Methods

Production of Antisera. Antisera to group B and group C streptococcal carbohydrates were raised in Monash University laboratory rabbits by hyperimmunization with killed vaccines of either *Streptococcus agalactiae* (group B) or *Streptococcus equi* (group C) (Osterland et al., 1966).

Purification of Antibodies. Antibodies to group B and group C streptococcal carbohydrates were prepared from hyperimmune sera using streptococcal whole cell immunoadsorbents. Whole streptococcal cells, pepsin treated to remove the outer protein layer and expose the group specific carbohydrate (Osterland et al., 1966), were suspended in 5% formal saline for 2 h at room temperature, washed three times in saline, and finally resuspended in 0.01 M borate buffer, pH 7.2.

Immune serum was added to the immunoadsorbent at a concentration of 2 mg of IgG per mL wet-packed immunoadsorbent. Gentle mixing was applied for 30 min at 37 °C and the mixture left at 4 °C overnight. The immunoadsorbent was removed by centrifugation at 5000*g* for 15 min at 4 °C and resuspended in ice-cold saline. The immunoadsorbent, containing bound antibody, was washed three times with ice-cold saline and extracted with 5 mL of 15% NaCl for 1 h in the presence of a drop of toluene. The immunoadsorbent was collected by centrifugation and reextracted with a further 2 mL of 15% NaCl. The NaCl extracts were combined, dialyzed against 0.15 M saline, and concentrated to approximately 2 mg/ml IgG.

Reduction and Alkylation of IgG. Purified IgG was extensively reduced and alkylated in the presence of 6 M guanidine hydrochloride by the method of Small and Lamm (1966). Guanidine was removed from the completely reduced and alkylated material by extensive dialysis against distilled water.

Discontinuous Polyacrylamide Gel Electrophoresis. The completely reduced and alkylated IgG was electrophoresed in 4% polyacrylamide gels by a modification of the alkaline urea method of Reisfeld and Small (1966). The sample (300 μg) was applied in 6 M urea and 20% sucrose directly onto the running gel described by Reisfeld and Small.

After electrophoresis, gels were stained in Coomassie brilliant blue R250 and destained electrophoretically.

Radioiodination and Trypsin Digestion of Isolated Light Chains. Stained L chain bands in polyacrylamide gels were

TABLE I: Antibody Origin and Variable Peptide Content of Isolated Rabbit L Chains.

Rabbit No.	Antibody Specificity	b Allotype	L Chains Isolated	Variable Peptides Obsd
4	B-CHO[a]	b_4b_5	4a	5, 7, 8, 9, 10
			4b	5, 7, 9, 10, 11
9	B-CHO	b_4b_5	9a	6, 7, 9, 10, 11, 12, 13
25	B-CHO	b_4b_4	25c	5, 7, 8, 9, 11
26	B-CHO	b_4b_4	26a	5, 7, 8, 9, 10, 11
			26b	5, 7, 8, 9, 10, 12
144	B-CHO	b_4b_4	144a	5, 7, 8, 10, 12
			144b	5, 7, 8, 9, 10, 11
392	C-CHO[a]	b_4b_5	392a	5, 6, 7, 10, 11, C
			392c	5, 7, 8, 9, 10, 11, 12, C
			392d	5, 6, 8, 9, 10, 11, C
532	C-CHO	b_4b_4	532a	6, 7, 9, 10, 11, 12, C
			532b	5, 6, 9, 10, 12, C
			532c	5, 7, 9, 10, 11, 12, C

[a] B-CHO, group B streptococcal carbohydrate; C-CHO, group C streptococcal carbohydrate.

excised, eluted, and iodinated with $Na^{125}I$ (Cat. No. IMS 30, The Radiochemical Centre, Amersham, Bucks) by the methods described by Bray and Brownlee (1973). After iodination, 1 mg of rabbit γ-globulin was added as a carrier and the protein in 1.5 mL of 1% NH_4HCO_3 digested with 20 μL of trypsin solution (1 mg/mL in 0.05 M Tris[1]-HCl, pH 7.4) for 16 h at 37 °C. Previous studies have shown tryptic digestion of rabbit L chains to be complete within 8–10 h (Small et al., 1965).

Residual radioactive contaminants were removed by passing the digest through a Sephadex G-25 column as described by Bray and Brownlee (1973).

Tryptic Peptide Maps. Peptide maps of trypsin digested ^{125}I-labeled L chains were prepared on Whatman 3MM chromatography paper. Separation in the first dimension was by electrophoresis at pH 3.6 (10% acetic acid–pyridine) for 2 h at 3000 V. Descending chromatography in butanol–acetic acid–pyridine–water (90/18/60/72 v/v) was carried out at 90 °C to the first dimension. The dried peptide maps were exposed to x-ray film for 12 to 18 h.

Results

Preparation of Antibody Fractions. Antibodies were prepared from hyperimmune sera by adsorption and elution from immunoadsorbents specific for either anti-group B or anti-group C streptococcal antibodies. The allotype and antigen binding specificity of the purified antibody fractions is given in Table I.

Isolation of L Chains by Polyacrylamide Gel Electrophoresis. The completely reduced and alkylated antibodies were subjected to electrophoresis in polyacrylamide gels at pH 9.4 in 10 M urea. Representative electrophoretic patterns are shown in Figure 1. Heavy chains remained at the cathodal end of the gel, while the L chains separated into several discrete bands as they migrated toward the anode. The L chain bands were labeled according to the serum number and the position of the L chain in the gel. These are listed in Table I.

Tryptic Peptide Maps of ^{125}I-Labeled L Chains. Light chain bands were excised from acrylamide gels, the protein eluted and, after labeling with ^{125}I, digested with trypsin (Bray and Brownlee, 1973). Radioiodination using Chloramine-T introduces ^{125}I at tyrosine residues in the polypeptide chain (Hunter and Greenwood, 1962). Therefore only tryptic peptides containing tyrosine were seen in the peptide maps. A peptide map prepared from ^{125}I labeled, trypsin digested L chains is shown in Figure 2. Tryptic peptide maps prepared at different times from the same L chain band in polyacrylamide gels yielded identical peptide patterns. Complete labeling and digestion and adequate resolution are indicated by this pattern because rabbit light chains contain 10–11 mol of tyrosine per mol of L chain (Cohen and Porter, 1964; Koshland et al., 1966) and this number of labeled peptides was observed. Peptide maps of 14 L chains, derived from 7 antisera (Table I), were prepared in this way.

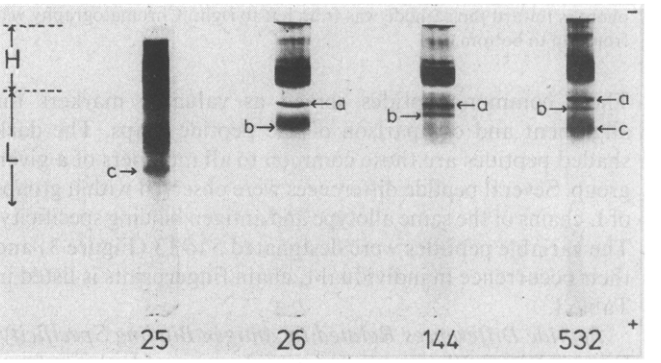

FIGURE 1: Disc electrophoresis in alkaline urea–polyacrylamide gels of 300 μg of completely reduced and alkylated anti-streptococcal antibodies. H, heavy chains, L, light chains; migration was toward the anode.

The L chains from which the peptide maps were prepared fell into four groups based on the antigen binding specificity and allotype of the antibodies from which they were derived. By comparing tracings of the original x-ray films, composite peptide maps were prepared for each of these groups (Figures 3a to 3d). Peptides 1 to 4 were found to be common to all the L chains examined and probably represent C region peptides and invariant peptides of the V region; i.e., peptides lying outside the hypervariable regions (Wu and Kabat, 1970).

[1] Abbreviation used: Tris, tris(hydroxymethyl)aminomethane.

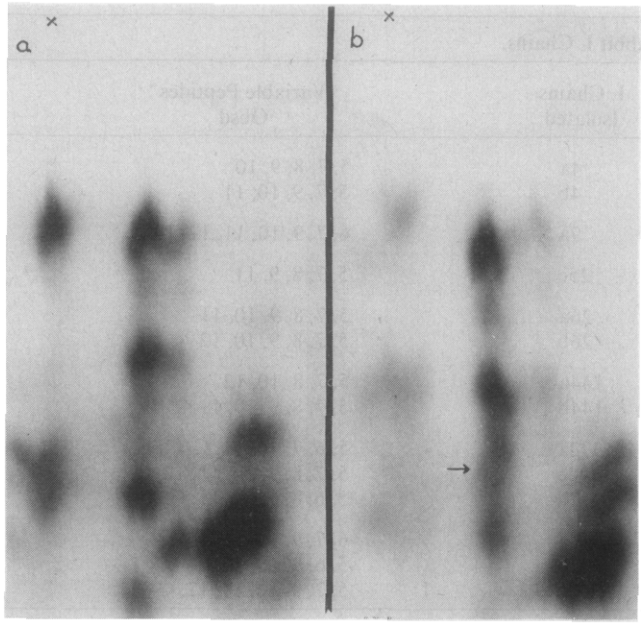

FIGURE 2: Autoradiograms of tryptic peptide maps of ^{125}I-labeled L chains. (a) L chain 26b; (b) L chain 392a. Peptide "C" is shown by the arrow. The point of application of the digests is indicated by X. Electrophoresis toward the cathode was from left to right. Chromatography was from top to bottom.

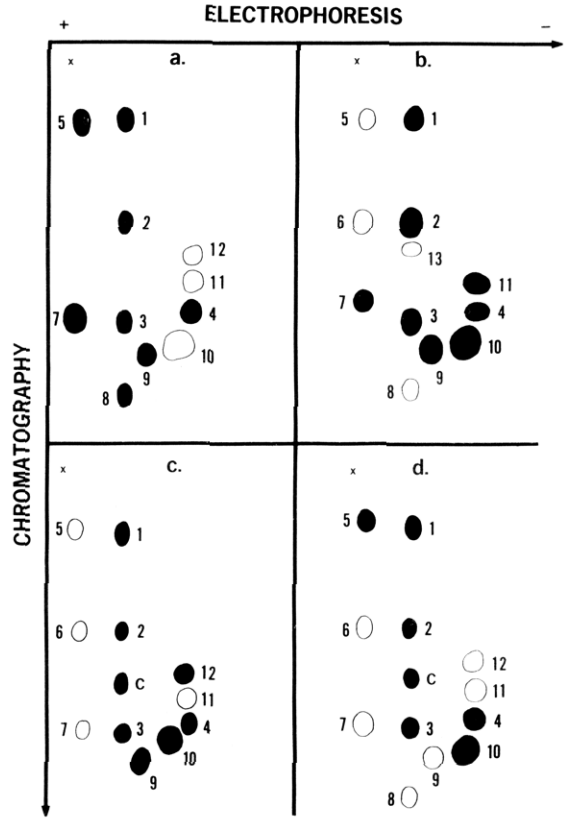

FIGURE 3: Diagrammatic representations of composite tryptic peptide maps of L chains from (a) b_4b_4 antibody to group B streptococcal carbohydrate; (b) b_4b_5 antibody to group B streptococcal carbohydrate; (c) b_4b_4 antibody to group C streptococcal carbohydrate; (d) b_4b_5 antibody to group C streptococcal carbohydrate; peptides 1 to 4 are common to all L chains. The dark shaded peptides are common to all L chains of a given antigen specificity and allotype group. Variable peptides are numbered 5 to 13.

These common peptides served as valuable markers for alignment and comparison of the peptide maps. The dark shaded peptides are those common to all members of a given group. Several peptide differences were observed within groups of L chains of the same allotype and antigen binding specificity. The variable peptides were designated 5 to 13 (Figure 3) and their occurrence in individual L chain fingerprints is listed in Table I.

Peptide Differences Related to Antigen Binding Specificity. Examination of the composite peptide maps of L chains from antibody with specificity for group B or group C streptococcal carbohydrates revealed a peptide present in L chains derived from anti-group C antibody that was not present in L chains derived from anti-group B antibody. This peptide was designated "C". Light chains from anti-group B antibody did not possess any ^{125}I-labeled peptides that were not present in L chains from anti-group C antibody. With the exception of peptide "C" the differences observed between peptide maps of L chains derived from antibodies of different antigen binding specificity appeared no greater than the differences observed among L chains from antibodies of the same specificity.

Control extractions of group-C streptococcal immunoadsorbent alone failed to yield detectable protein. Thus, peptide "C" does not arise from this possible source.

Allotype Related Peptides. Comparison of composite maps of L chains of the same antigen binding specificity but derived from either homozygous (b4b4) or heterozygous (b4b5) antibodies did not reveal any peptides with apparent allotype specificity. Allotype could not be assigned to L chains derived from heterozygous (b4b5) antibody as the reduced and alkylated L chains do not react with anti-allotype sera.

Peptide Maps of L Chains Derived from Laboratory and Australian Wild Rabbits. Antibody 9, with specificity for group B carbohydrate, was obtained by the immunization of an Australian wild rabbit. When compared with L chains derived from antibody elicited in laboratory rabbits against the same antigen, L chains from antibody produced in an Australian wild rabbit showed many similarities in the tryptic peptides observed (Table I). Antibody 9 L chains shared eight common peptides (1, 2, 3, 4, 7, 9, 10, 11) with L chains of the same allotype and antigen binding specificity derived from laboratory rabbits. However, 9a L chains possessed one tryptic peptide that was not found in the peptide maps of other L chains (peptide 13).

The Occurrence of L Chains with Identical Peptide Maps. Of the 14 L chains examined by the radioiodinated, tryptic peptide map technique, two L chains, 26a and 144b, possessed identical peptide maps consisting of 10 radioiodinated peptides (Table I).

Discussion

Comparison of peptide maps prepared from 14 radioiodinated L chains derived from rabbit anti-streptococcal antibodies allowed the identification of an ^{125}I labeled peptide apparently linked to the antigen binding specificity of the antibody from which the L chains were prepared. This peptide (designated C) was present in L chains from anti-group C streptococcal antibodies, but absent in L chains from anti-group B streptococcal antibodies. Peptide C obviously contains tyrosine, as it is at this residue that iodination occurs. The presence of tyrosine in a peptide implicated in the antibody combining site of antibody to group C streptococcal carbohydrate is supported by the identification of this residue in the combining sites of antihapten antibodies by affinity labeling techniques (Pressman et al., 1970; Grossberg and Pressman, 1975). As peptide C appears in the tryptic peptide maps of all the L chains derived from anti-group C streptococcal antibody,

it must be of a relatively similar composition in each of these polypeptide chains. Sequence analysis of peptides linked to the definition of the antigen binding site may provide valuable information with respect to the structure of the site while avoiding the necessity for complete V region analysis.

No allotype related peptide differences were observed in the peptide maps of the L chains examined. The carboxy-terminal sequence of the rabbit L chain is characteristic of its b allotype specificity (Appella et al., 1969). Tryptic digestion would yield two peptides that contain some of the allotype specific residues. However, the available sequence data indicate that neither of these peptides contains tyrosine and would therefore not be visualized by the ^{125}I technique. The results obtained were consistent with this expectation.

Light chain 9a, prepared from antibody produced in an Australian wild rabbit, contained one peptide not seen in any of the L chains derived from Monash laboratory rabbits. However, the wild rabbit L chains do contain the four peptides common to all the other L chains studied and thus assumed to be derived from the C region. Thus, the peptide difference observed most likely reflects a structural difference in the VL regions of wild and laboratory rabbits. This may be due to the widely differing selective pressures to which these two groups of rabbits have been subjected.

The occurrence of 2 L chains with identical ^{125}I-labeled tryptic peptide maps indicates a high degree of sequence homology of their V regions. Each L chain yielded ten ^{125}I-labeled peptides, four of which probably represent constant region peptides. The remaining six peptides must originate from the V region. Sequence data (Margolies et al., 1975; Jaton, 1975) indicate that both the first and third hypervariable regions of the rabbit L chain contain tryptic peptides that possess tyrosine and will therefore appear as ^{125}I-labeled peptides. This finding therefore provides evidence for the existence of identical VL regions in antibodies derived from separate rabbits. Montgomery et al. (1975) have recently reported the identity of the combining sites of two antibodies to DNP obtained from separate rabbits. Sequence analysis of L chains from six rabbit homogeneous antibodies to group A variant streptococci (Braun et al., 1975) has shown identity in the N-terminal amino acid sequence to residue 42 in five of these chains. These L chains were produced by closely related, but not inbred, rabbits.

The existence of V regions with a high degree of sequence homology, induced by the same antigen, in two or more members of an outbred species such as the rabbit, is indirect evidence that these V regions are coded by genes carried in the germ line. Although identical V regions might also arise by somatic mutation in several members of such an outbred species, a very restricted mechanism of mutation and selection would need to be invoked to account for such an event.

The set of techniques used in this study provides a rapid, reliable and sensitive means of assessing relatedness among polypeptides which are present in relatively low concentration. This approach should prove useful in investigations of membrane proteins which can be radioiodinated (Marchalonis et al., 1971) but are difficult to obtain in sufficient quantity to enable extensive sequence analysis by any present method.

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