ANTIBODY RESPONSE TO THE STREPTOCOCCAL GROUP A-VARIANT POLYSACCHARIDE IN BASILEA RABBITS LACKING κ-POLYPEPTIDE CHAINS

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The height of immune responses in rabbits to the streptococcal group polysaccharides A, A variant, and C is genetically under polymorphic control (1, 2). The molecular nature of regulation of antibody levels upon hyperimmunization with bacterial vaccines has not yet been determined. In inbred mice, where an analogous polymorphism has been found it appears that several genes exert an influence on antibody levels (3, 4). Normal pools of rabbit immunoglobulins contain 90–95% κ-polypeptide light chains (5, 6). Specific antibodies are mainly constituted of κ-chain molecules, and λ-chains have not been found to participate significantly in antibody responses of restricted heterogeneity.

In studying the role of λ-type chains in immune responsiveness a new strain of rabbits can now be used (7). This variant strain, called BASILEA, was derived from a mutant lacking the expression of κ-polypeptide chains; in it κ-chains are replaced by λ-chains. The marker, called *bas*, is an allelic form of the *b* locus (7). Heterozygotes (e.g. *b4/bas*) show normal κ/λ-ratios (7).

It was found that *bas* homozygotes are able to respond with very high antibody levels. Despite dominance of λ-polypeptide chain-containing antibodies, the overall pattern of anti-streptococcal group A-variant polysaccharide (Av-CHO)[1] antibodies is of similar complexity in BASILEA rabbits as in selectively bred rabbits expressing mainly κ-chain-containing antibodies.

Materials and Methods

Rabbits. BASILEA rabbits were used; four of these rabbits were heterozygous (*b4/bas* or *b5/bas*) and seven were homozygous for *bas*. At the heavy chain variable (*V*) region locus, 8 rabbits were *a3/a3* homozygous and three rabbits were heterozygous (*a1/a3*) (see Table I).

Immunization, Determination of Antibody Response, Isolation of Restricted Antibodies and of Polypeptide Chains. The immunization with group A variant streptococci (strain A486 variant M-), the Farr assay for the determination of antibody levels, microzone electrophoresis, analytical isoelectric focusing (IEF), the classification of the heterogeneity of the response, isolation of clonal antibody by preparative agarose block electrophoresis, and the separation of partially reduced and alkylated antibody into light and heavy polypeptide chains all have been described previously (8–13).

Peptide Maps of Isolated Light Chains. Partially reduced and alkylated light chains of stan-

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[1] *Abbreviations used in this paper*: Av-CHO, streptococcal group A variant polysaccharide; dom, dominant; IEF, isoelectric focusing; ME, microzone electrophoresis; PBS, phosphate-buffered saline; V domains, variable domains of antibodies.

dard rabbit IgG (Pentex Biochemical, Kankakee, Ill.), pooled IgG, and chains from the major Av-CHO-binding clonotype of rabbit BS-4433 (Table I) were completely reduced in 7 M guanidine-HCl, containing 0.4 M Tris (13), pH 8.2, and alkylated first by using ^{14}C-iodoacetic acid (Amersham/Searle Corp., Arlington Heights, Ill.), followed by excess cold iodoacetic acid. After dialysis against 0.05 M NH_4HCO_3, the fully reduced and alkylated light chains were digested for 4 h at 37°C with TPCK-treated trypsin (Merck AG, Darmstadt, W. Germany) at a protein enzyme ratio of 100:2. After lyophilization, the trypsin-digested light chains were redissolved in 1 M NH_3 and freed from undissolved material by centrifugation. Peptide maps (14) were performed on 20 × 20 cm cellulose thin layer plates (Merck AG). Chromatography was carried out first for 10–12 h (25 ml pyridine, 37.5 ml *n*-butanol, 7.5 ml acetic acid, and 30 ml H_2O), followed by electrophoresis on a cooled plate at 800 V for 60 min in the second direction (buffer: 22 ml pyridine and 32 ml acetic acid were brought up to 2 liters with H_2O). Thin layer plates were then stained with fluram (Roche Diagnostics Div., Hoffmann-La Roche, Inc., Nutley, N. J.) by using 25 μg/ml in 0.5% pyridine aceton solution (15). Since spots fade within the subsequent 2 days, they were marked at the back of the plate, dried, and autoradiographed (AGFA-Gevaert, Osray M3).

Determination of Light Chain Type of Specific Antibody. For the subsequent methods to be described a newly identified rabbit λ-chain allotype, called L22, was used (S. Weiss, and A. S. Kelus, unpublished observations). This marker has so far been found in all BASILEA rabbits (>200 tested).

To determine directly the light chain type of specific antibodies in antigen binding, antisera of *bas* heterozygous and homozygous rabbit immunoglobulins were first precipitated by anti-λ allotype antisera (specificities c7, c21, and L22) in Ouchterlony double diffusion analyses at equivalence (16, 17). The plates were then washed extensively, and ^{125}I-labeled Av-CHO was diffused from the central antiserum well into the immune precipitate (18). Plates were washed, dried, autoradiographed, and stained.

The peak antibody fraction to Av-CHO of rabbit BS-4433 was quantitated for its content of λ-chain by a modified ethylchloroformate method (19). The data were compared with a simultaneously analyzed b4 Av-CHO-specific homogeneous antibody (K49-501).

Results

Response Patterns of BASILEA Rabbits to the Streptococcal Av-CHO. Table I summarizes the primary and secondary immune responses to the Av-CHO in 11 rabbits. With regard to antibody levels only the highest titers are given. Out of these 11 rabbits 4 were heterozygous at the light chain locus, and in addition 3 rabbits were heterozygous at the variable region heavy chain locus. The antibody levels to the Av-CHO ranged from 0.6 to 34.0 mg/ml of serum; however, the majority of rabbits had levels of greater than 10 mg/ml. When the criteria for classifying the anti-streptococcal group polysaccharide responses by microzone electrophoretic analysis were applied (10), three rabbits were regarded as heterogeneous responders, six rabbits showed a restricted, and two rabbits a monoclonal, response (Fig. 1).

As shown previously, selectively bred rabbits express mainly κ-light chains in anti-streptococcal group polysaccharide antibodies (20–22). The Av-CHO-specific antibody in the antisera of BASILEA rabbits nearly always accounted for more than 90% of the immunoglobulin. This ratio of specific to unspecific immunoglobulin is analogous to that in κ-chain-expressing rabbits (23). IEF analysis of BASILEA antisera allowed correlation of the Na_2SO_4 precipitable and bromphenol blue stainable bands with binding specificity for Av-CHO. The only exception was antiserum BS-4432, where the major clonotype (isoelectric point 6.1) did not bind Av-CHO. Similar observations were previously reported showing that dominant clonotypes elicited by immunization with streptococcal vaccines are not always group polysaccharide-specific antibodies (24, 25). It ap-

TABLE I

Basilea Rabbits: Heavy and Light Chain Allotypes, Antibody Levels to Av-CHO, and Number of Clonotypes of Av-CHO-Specific Antibody

Rabbit no.		Allotypes		Av-CHO specific			
		Heavy chain	Light chain	Antibody levels	Response (ME)	Bands (IEF)	Clonotypes
				mg/ml			
1	BS 4171	a1/a3	b4/bas	9.0	Restricted	51	17
2	BS 4174	a1/a3	b4/bas	17.4	Monoclonal	53	17–18
3	BS 4176	a1/a3	b4/bas	10.8	Heterogeneous	50	16–17
4	BS 4300	a3/a3	bas/bas	5.9	Restricted	38	12–13
5	BS 4301	a3/a3	bas/bas	13.3	Heterogeneous	53	17–18
6	BS 4315	a3/a3	bas/bas	15.6	Restricted	36	12
7	BS 4323	a3/a3	bas/bas	34.0	Restricted	39	13
8	BS 4325	a3/a3	bas/bas	11.9	Heterogeneous	48	16
9	BS 4345	a3/a3	b5/bas	13.0	Restricted	34	11–12
10	BS 4432	a3/a3	bas/bas	0.6	Restricted	30	10
11	BS 4433	a3/a3	bas/bas	31.2	Monoclonal	33	11

pears that a substantial number of clonotypes expressed at a low quantitative level exhibited a high antigen binding capacity, for example, the dominant clonotype of BS-4433 (pH 6.7–7.0) was compared with the discrete clonotype focusing at pH 7.5–7.9. Furthermore, the number of Av-CHO-binding bands expressed by *bas/bas* rabbits was of the same order as the one elicited in *b4/bas* rabbits, e.g. patterns of BS-4171 and BS-4325 (Fig. 2, Table I). This number of bands was not critically different in rabbits heterozygous at the heavy chain *a* region and for *bas* (e.g. BS-4171). In addition, the number of Av-CHO-binding clonotypes was also of the same order in rabbit antisera expressing mainly κ-light chains.

Finally, as was expected from previous work with antibodies of closely related rabbits (8), substantial pattern overlapping was observed (Fig. 2).

Classification of the Av-CHO-Specific Responses. The data shown here may be used to re-evaluate the previous notations of monoclonal, restricted, and heterogeneous responders to streptococcal group polysaccharides (10). According to this classification, based on quantitative analysis by microzone electrophoresis (ME) patterns, an antiserum was referred to as monoclonal if the protein under the peak of the densitometric tracing of the ME accounted for more than 60% (e.g. BS-4433, Fig. 1). If such a peak accounted for 30–60% of the immunoglobulin area, the response was called restricted (e.g. BS-4315, Fig. 1); finally, if a peak accounted for less than 30% (BS-4325, Fig. 1) the response was scored as heterogeneous.

On the basis of the IEF patterns for the *bas* heterozygous and homozygous rabbits demonstrated here (Fig. 2), and also for normal κ-chains expressing rabbits, such a distinction is by qualitative terms untenable: the number of different antibodies activated by the antigen, hence the repertoire of genes available in the various rabbits, is similar.

The Light Chain Type of Av-CHO-Binding Antibodies. To analyze the light

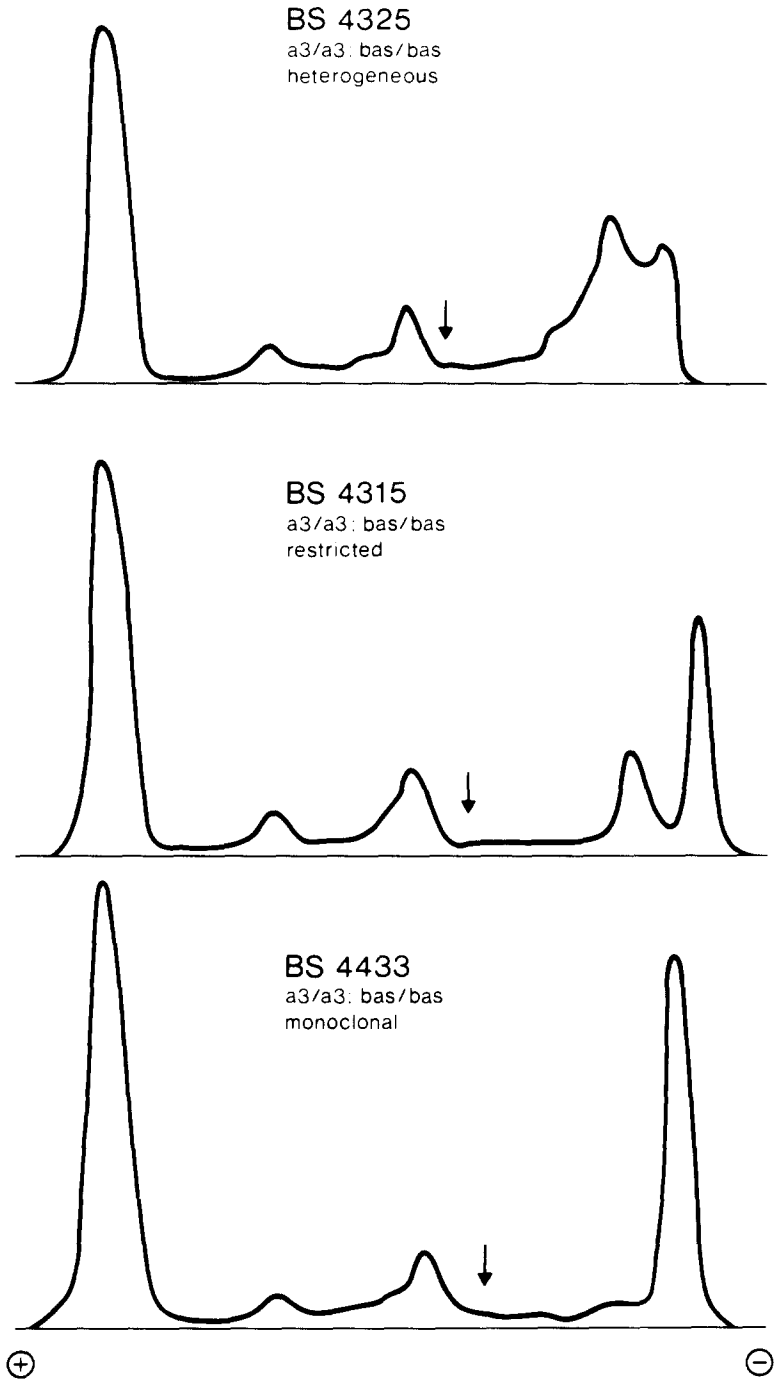


FIG. 1. Densitometric tracings of typical ME patterns of anti-streptococcal Av-CHO antisera from BASILEA rabbits.

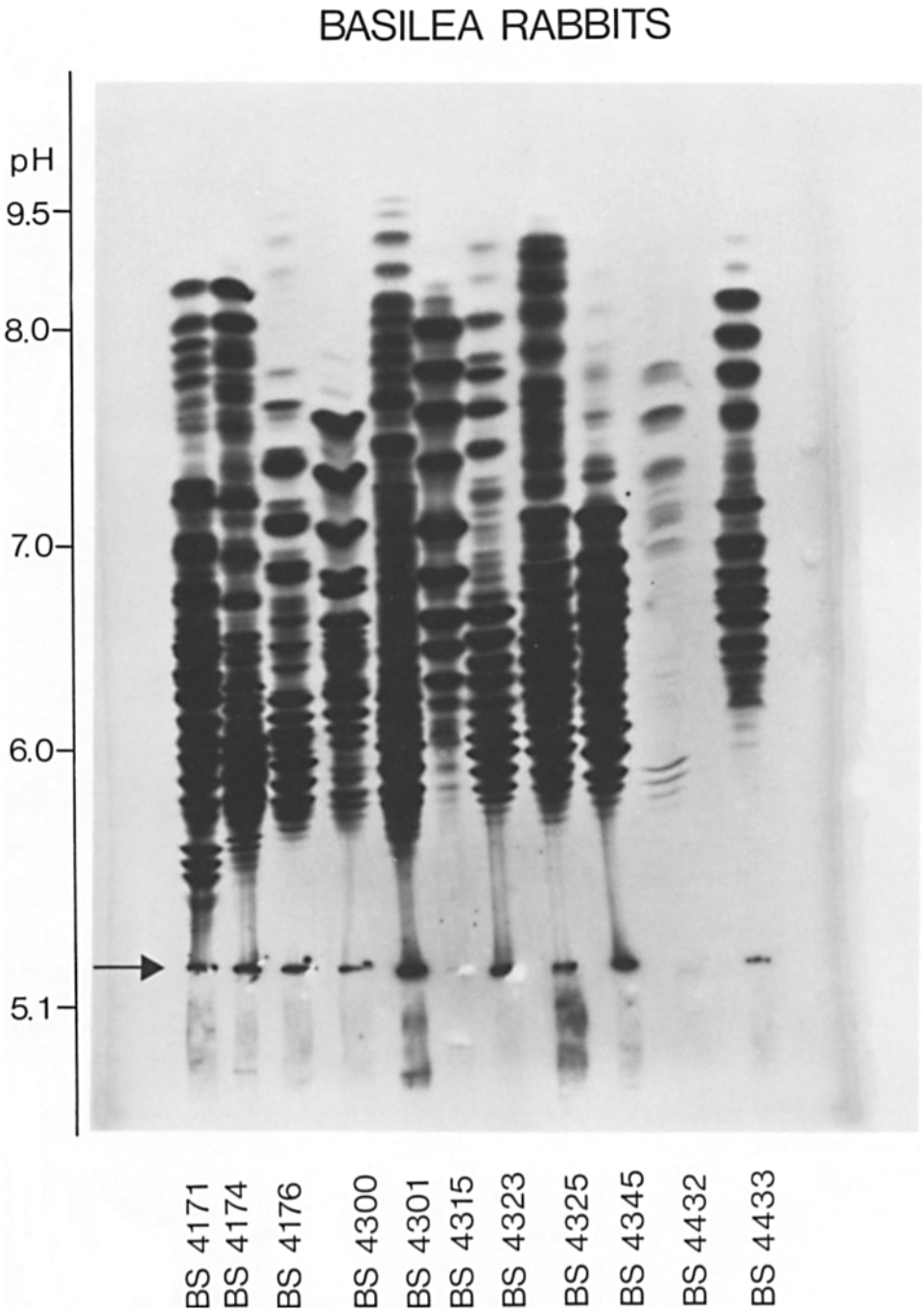


FIG. 2. Analytical IEF patterns of streptococcal Av-CHO specific antibodies from BASILEA rabbits developed by binding of ^{131}I-Av-CHO and autoradiography (8). The film was overexposed to reveal also the minor clones in serum BS-4432.

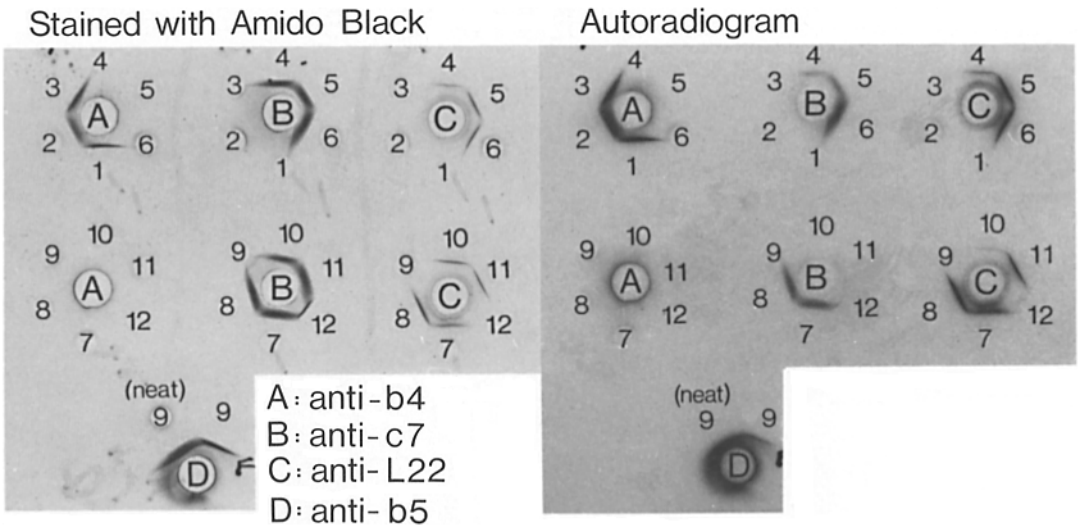


FIG. 3. Ouchterlony double diffusion analysis for determination of light chain type of anti-Av-CHO antibodies. Sample 1–11 are antisera of BASILEA rabbits (for identification see Table I) diluted 1:10 with phosphate-buffered saline (PBS). Sample no. 12 is antiserum BS-4433 diluted 1:100 in PBS. Anti-c7 and L-22 precipitates of heterozygotes no. 1, 2, 3, and 9 were only weakly revealed in undiluted sera and thus not demonstrable in the patterns shown for clarity at 1:10 dilution.

chain type of Av-CHO-binding antibodies on a qualitative basis, sera of hyperimmunized rabbits were first precipitated by anti-b4 (κ), anti-b5 (κ), anti-c7 (λ), and anti-L22 (λ) antisera (S. Weiss, and A. S. Kelus, unpublished observations), and in a second step reaction ^{125}I-labeled Av-CHO was diffused into the pattern from the center wells. Fig. 3 demonstrates that polysaccharide-specific antibodies of *b4/bas* and *b5/bas* rabbits contain practically only b4 and b5 chains, respectively. Conversely, *bas/bas* rabbits express *only* λ-chain markers c7 and L22 in Av-CHO-binding antibodies. These qualitative data were fully supported by quantitative analysis performed with antibody isolated from the major peak of BS-4433 and a reference κ-light chain-containing clonotype K49-501 (Fig. 4). However, when Av-CHO-specific hyperimmune sera of 12 *b4* homozygous rabbits of a selectively bred rabbit colony were investigated for the expression of the c7, c21, and L22 markers by the radio double diffusion method, it appeared that all of these rabbits did express traces of λ-chains in specific antibodies. These were frequently associated with μ-chains (data not shown).

Map of Tryptic Peptides of Light Chain BS-4433. The dominant (dom) clonotype of antiserum BS-4433 (pH 6.7–7.0, Fig. 2), called BS-4433 dom Ab, was isolated with 85% purity (based on binding by an anti-c7 antiserum, Fig. 4) by preparative agarose block electrophoresis and re-electrophoresis. Because of the complexity of the IEF pattern of Av-CHO-binding antibodies anodal and cathodal of BS-4433 dom Ab, greater purity was not achieved by preparative electrophoresis. The light chain preparation of BS-4433 dom Ab was isolated with a 30% yield after partial reduction and alkylation. After complete reduction, alkylation, and digestion, the tryptic peptides were resolved into 20 distinct spots on a peptide map (Fig. 5 c). Five of these major spots carried ^{14}C-label

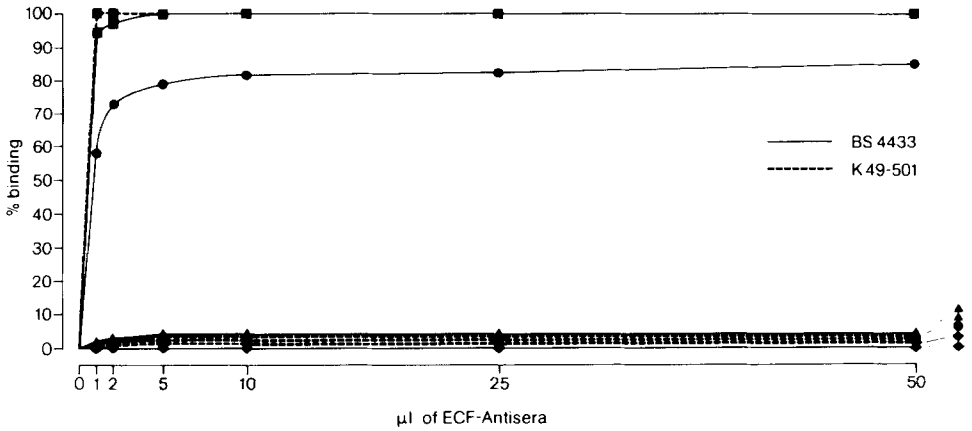


FIG. 4. Direct binding of the dominant clonotype of BS-4433 (bas) and K49-501 (b4) to anti-λ allotype sera insolubilized with ethylchloroformate. ●, anti-c7, ▲, anti-c21, ■, sheep anti-rabbit IgG, ◆, normal rabbit serum.

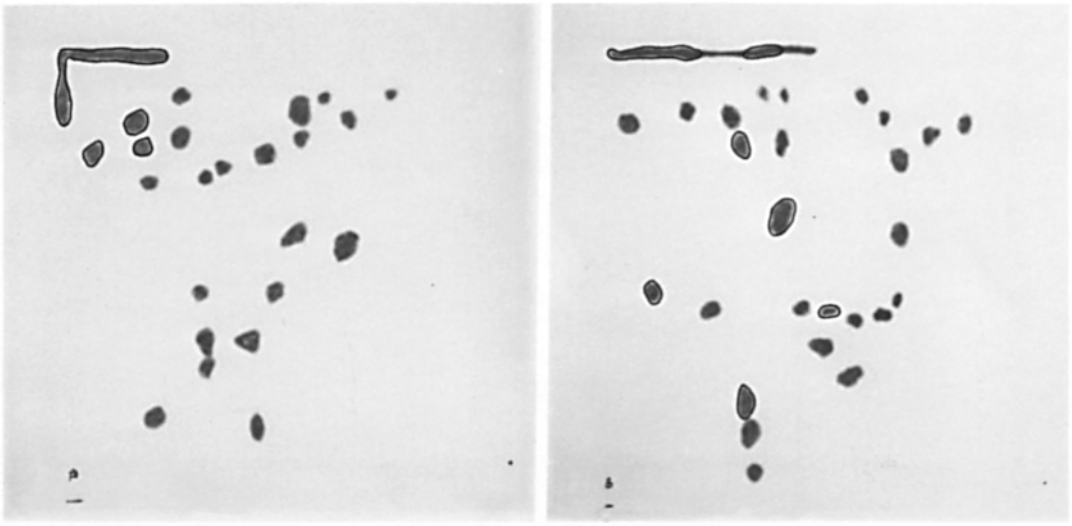
and thus marked the cysteine-containing peptides. This map resembled that one obtained with a pool of bas chains (Fig. 5 b, 13). However, it was very different from the map of light chains (Fig. 5 a) isolated from standard rabbit IgG pool (Pentex Biochemical).

Discussion

Rabbits of the BASILEA variant were analyzed for the magnitude of the immune response to the streptococcal group Av-CHO. The majority – five out of seven – of the *bas/bas* rabbits responded with high antibody levels to this bacterial polysaccharide. In addition, the four rabbits of this family, heterozygous for the light chain locus, *b4/bas* or *b5/bas*, responded with high antibody levels in a manner similar to offspring of a cross between a BASILEA male and female rabbits from a colony bred selectively for restricted high responders to this antigen (8, data not shown). While all heterozygous high responders produced predominantly antibodies containing κ-chains, with a very small proportion of λ-chain associated antibodies, *bas* homozygotes produced antibodies with λ-chain. The evidence that these light chains are of λ-type is serological (markers c7, c21, and L22); also it is chemical by the peptide map and amino acid analysis of a single isolated antibody (BS-4433), and by preliminary amino acid sequence data.

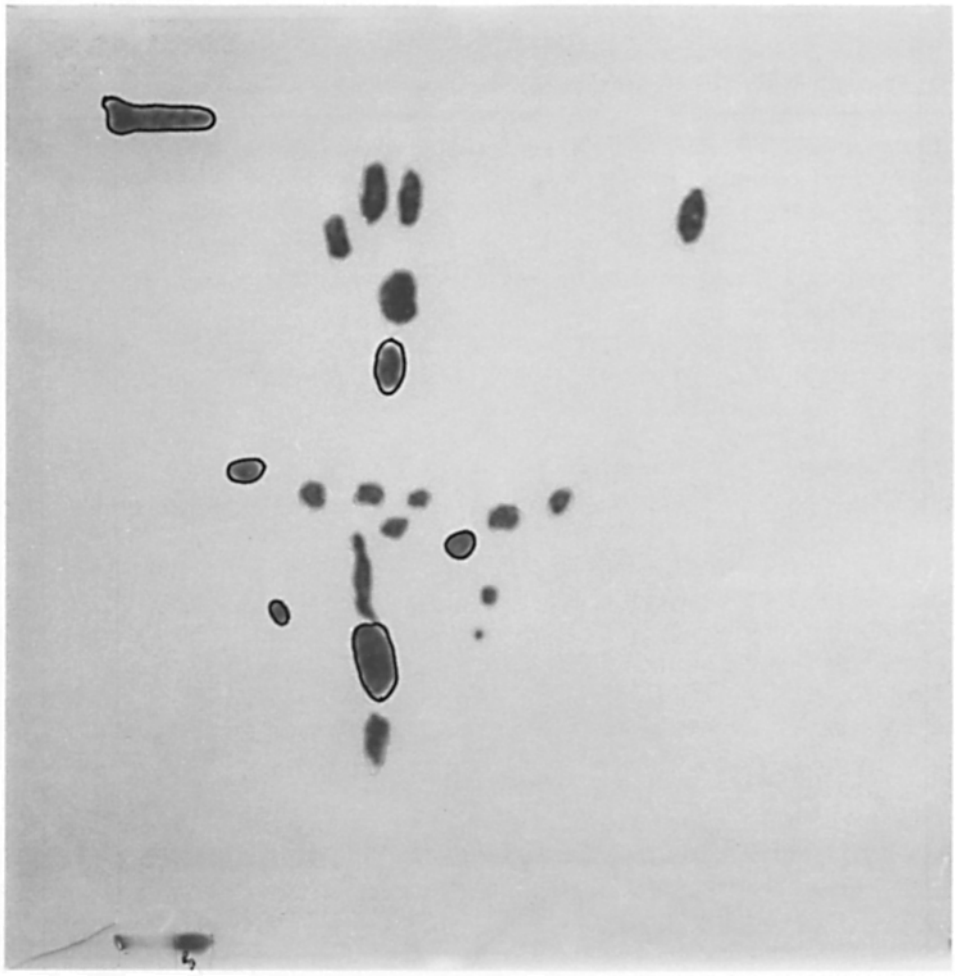
The high incidence of restricted high responders in BASILEA rabbits is presumably due to a founder effect. That is, the original male identified as a mutant (founder) must have been, by accident, a restricted high responder. Thus, the restricted response phenotype was unintentionally introduced into our new variant strain of rabbits. Since the founder male is the source of over 50% of the genetic material of the strain, any genes introduced with him are of necessity at high frequency. There is no relationship between the phenotype of restricted high responders and the lack of κ-chain expression.

In rabbits, λ-chains account for 5–10% of the light chain pool (5, 6). In immunized rabbits of the BASILEA strain, that are *bas* heterozygotes (e.g. *b4/*



a Standard rabbit pool (pentex)

b bas pool



C BS-4433 dominant clonotype

bas), little of the anti-Av-CHO response is due to antibodies containing λ-chains, i.e. κ-chains dominate the response as in normal rabbits. On the other hand, homozygotes *bas/bas* are just as capable of mounting analogous anti-Av-CHO antibody responses of restricted heterogeneity. It should be added that immunization of *bas/bas* rabbits with a variety of antigens leads to immune responses which are completely analogous to those of normal rabbits (S. Weiss, and A. S. Kelus, unpublished observations).

How restricted is the anti-Av-CHO response in these homozygotes, and is it restricted because the repertoire of structural genes available for λ-variable regions is smaller than that for κ-chains? This, incidentally, is the argument for an analogous situation in BALB/c mice where anti-dextran antibodies are associated exclusively with the expression of λ-chain containing antibodies (26). The analysis of antisera of *bas/bas* rabbits by IEF and specific antigen binding reveals that the degree of heterogeneity is of the same order in both *bas* heterozygotes and homozygotes. Furthermore, the anti-Av-CHO response in normal rabbits of selectively bred colonies (8) is of no greater heterogeneity than that of *bas* heterozygotes or homozygotes. This degree of heterogeneity is also of the same order in heterogeneous high or low responders, where 20–25 Av-CHO clonotypes per rabbit were counted (27).

We would like to emphasize that, although useful for practical descriptive purposes, the terms monoclonal, restricted, and heterogeneous fail to correctly describe the number of structural gene products identified in monoclonal and restricted responses as opposed to heterogeneous responses. The number of phenotypically-expressed clonotypes is similar at a qualitative level but differs in quantitative terms. Hence, the terms monoclonal, restricted, and heterogeneous relate only to levels of expression. However, these terms give no information with regard to the number of structural genes available for a given antigen. More importantly, with repeated stimulation, clonotypes expressed at low and at high levels coexist just as much in bas rabbits and normal rabbits (18). According to these findings, it is the previous history of the animal that determines clonal hierarchies (28). Clonal dominance in this situation is frequently achieved by low affinity antibodies, and it is maintained during repeated immunizations (W. Schalch, and D. G. Braun, unpublished observations).

The data reported here for a variant strain of rabbits expressing only λ-type chains when hyperimmunized against a group polysaccharide of streptococci support previous findings. They also suggest that the λ-chain repertoire is not smaller than the κ-chain repertoire of rabbits.

Summary

Rabbits from a variant strain called BASILEA, in which homozygotes express only λ-type chains and heterozygotes have normal κ/λ ratios, were hyperimmu-

FIG. 5. Tryptic peptide maps of fully reduced and alkylated ^{14}C-light chains from: (a) pooled standard rabbit IgG, (b) pooled *bas* IgG, (c) dominant clonotype of BS-4433. First direction (chromatography) from top to bottom, second direction (electrophoresis) from left to right. Spots were developed with fluram and marked on the backside. Radioactive peptides were marked by circles.

nized with a streptococcal group A variant vaccine. Homozygotes (*bas/bas*) produced antibodies with λ-chains, heterozygotes, however, produced predominantly antibodies with κ-chains. The incidence of restricted high responders in the BASILEA strain was high; it was probably introduced by the original mutant rather than by the loss of κ-chains (founder effect). The degree of heterogeneity of homozygotes is similar to the heterogeneity of heterozygotes, and to that of rabbits expressing κ-chains. This suggests that in the rabbit, the repertoire of λ-chain genes is of similar size to that of κ-chain genes.

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