Brief Definitive Report

PAUCITY OF PHOSPHORYLCHOLINE-SPECIFIC CLONES IN B CELLS EXPRESSING THE V_HT15 GENE PRODUCT

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Despite the considerable potential diversity of the immune system, the antibody responses to certain antigens are encoded by a single V_H gene, one J_H and few V_L segments only (1–4). This phenomenon of clonal dominance can either result from selective forces that expand certain clones before antigen encounter or, alternatively, from the nature of the antigen that singles out a clone that is not particularly overrepresented in the preimmune repertoire. To discriminate between these two possibilities it is necessary to select clones encoded by a given V_H and analyze their specificity. To obtain a representative collection of hybridomas induced and selected on the basis of the expression of the V_HT15 gene product, we injected mice with the anti-V_HT15 (TC54) (5) mAb coupled to LPS and fused their spleens with the SP2/0 nonsecretor myeloma. Analysis of 28 and 29 hybridomas, obtained from BALB/c and C.B20 mice, respectively, revealed that none of the BALB/c and only two of the C.B20 clones recognize PC antigens. IEF studies also revealed the possible existence of highly preferential V_H–V_κ segment associations.

Materials and Methods

Animals and Immunization. BALB/c and C.B20 mice were obtained from the colony of the Institut Pasteur (Paris, France). LPS from *Salmonella typhimurium* was obtained from Difco Laboratories Inc. (Detroit, MI). The purified TC54 (anti-V_HT15) mAb was copolymerized with LPS as previously described (6, 7). Mice were immunized intraperitoneally with 10 μg of copolymer 3 d before fusion.

Proteins. The TC54 anti-V_HT15 mAb was kindly provided by Professor M. Scharff (Albert Einstein College, Bronx, NY), and its characteristics have been previously described (5). The AB 1.2 and GB 4.10 anti-T15 idiotype (8) proteins were obtained from Dr. J. F. Kearney (Cellular Immunobiology Unit, Birmingham, AL). All these reagents were isolated by affinity chromatography on T15 Sepharose-AH.

Generation of Hybridoma. Spleen cells from TC54-LPS-immunized mice were fused with SP2/0, plated in 24-well plates, and the hybridomas were prescreened for production of TC54-reacting molecules. All positive wells were cloned in 96-microwell plates and colonies were screened by the same method. One clone arising from each original well was selected and retested for the expression of the V_HT15 gene segment utilization by the lysate hybridization techniques (9).

DNA Probe and Hybridization. The 38CV probe, specific for members of the S107 family, is an agarose gel–purified restriction fragment of the 38C H^+ recombinant phage

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and was kindly provided by Dr. R. Perry (10) (Institute for Cancer Research, Philadelphia, PA). Hybridization was carried out essentially as described by Manser and Gefter (9).

Radioimmunoassays. Two kinds of RIAs were used in these studies. The first one consisted of a radioactive-binding inhibition. Briefly, clone supernatants were added to T15 (1 μg/ml), GB 4.10 (1 μg/ml, reference 8), AB 1.4 (1 μg/ml, reference 9), or F6(51) (γ1, κ; 1 μg/ml) precoated plastic wells together with ^{125}I-labeled TC54, T15 antibodies, or the anti-κ mAb H159.52.1. After an overnight incubation, the plates were washed and bound radioactivity was measured in a gamma counter.

The second assay consisted of a direct binding test. Hybridoma supernatants (100 μl) were added to plastic microwells precoated with DNP-BSA (20 μg/ml), PC-BSA (20 μg/ml), or lysozime (10 μg/ml). Thereafter, each well received ^{125}I-H159.52.1 protein and bound radioactivity was measured after 18 h of incubation at 4°C.

Two-Dimensional Gel Electrophoresis. Two-dimensional gel electrophoresis was carried out by a modification of the procedure described by Perlmutter et al. (11). Cloned hybridomas cells were washed twice in balanced salt solution and cultured for 30 min at 10^7 cells/ml in leucine-free medium containing glutamine and antibiotics. Thereafter, each culture received 300 μCi of L-4,5-[^{3}H]leucine (Amersham Corp., United Kingdom). Supernatants were collected after 3 h of incubation and proteins were precipitated with 10% final TCA in ice. H and L chains were separated by SDS-PAGE on a vertical flat bed apparatus with 6% acrylamide gel 1 mm thick.

Samples in 20 μl of SDS–sample buffer containing 300,000 cpm of [^{3}H]leucine were loaded into sample wells. ~10 μg of purified EF21 monoclonal protein (IgG1) labeled with fluorescein were included as migration markers. After electrophoresis the L chain band containing the fluorescent EF21 L chain was detected by exposing the gel to a UV light. A strip of the SDS-PAGE containing the L chains was excised and transferred to a tube containing 50 ml of equilibrium buffer (8 M deionized urea, 3% Triton X-100 and 5% 2-ME) and allowed to stand for 30 min at room temperature.

The IEF gel analysis was carried out as described by Perlmutter et al. (11).

Result and Discussion

Characteristics of V_HT15^+ Hybridomas. BALB/c and C.B20 mice were injected with LPS coupled to a mAb that recognizes the V_HT15 gene product independently of the L chain to which it associates. The hybrid B cell populations obtained were screened by means of the lysate hybridization technique (9) with a S107-specific DNA probe (10). Only those clones that both hybridized with the S107 probe and produced Igs that interact with the TC54 mAb antibody were retained for further analysis. This double selection made us confident that all clones retained indeed express the V_H15 gene product.

Table I and II show the characteristics of the 28 and of the 29 cloned hybridomas obtained from the spleen of BALB/c and C.B20 mice, respectively. All clones produce κ L chains and the H chain isotype of the vast majority of hybridomas belong to the IgM isotype.

The immune response of most inbred strains of mice to PC antigen is dominated by the utilization of a single V_H gene (V_HT15), three V_L, one J_H, and one J_κ segment (12, and reviewed in 13). The reason for this dominance is unclear, but it is possible that a disproportionate number of V_HT15 clones are devoted to combine with PC antigens. Analysis of our hybridoma collections clearly shows that this is not the case, since none of the BALB/c hybridomas and only two of the C.B20 clones display specificity for PC. Supernatants of C.B8.16.10 and C.B3.19.45 diluted to one-fifth incorporated 3,500 and 5,200 cpm, respectively, when tested for PC activity. The same signal was obtained with 150 ng of T15 protein diluted in medium. All other hybridomas incorporated <200 cpm even

TABLE I
Characteristics of BALB/c V_HT15^+ Hybridomas

Hybridoma	Isotype		Idiotypes*		Reactivity pattern				
	H	L	GB4.10	AB 1.2	PC	DNP‡	Ly§	RNP‖	HA¶
BA 30.23.3	μ	κ	−	−	−	−	−	−	−
BA 21.11.73	γ	κ	−	−	−	−	−	−	−
BA 29.13.40	μ	κ	−	−	−	−	+	−	−
BA 17.5.20	μ	κ	−	−	−	−	−	−	−
BA 29.12.10	μ	κ	−	−	−	−	−	−	−
BA 29.20.2	μ	κ	−	−	−	−	−	−	−
BA 12.14.58	μ	κ	−	−	−	−	−	−	−
BA 14.8.5	μ	κ	−	−	−	−	−	−	−
BA 22.20.16	μ	κ	−	−	−	+	−	−	−
BA 28.21.19	γ	κ	−	−	−	−	−	−	−
BA 20.16.1	μ	κ	−	−	−	+	+	+	−
BA 7.21.18	μ	κ	−	−	−	−	+	−	−
BA 23.22.4	μ	κ	−	−	−	−	−	−	−
BA 22.5.9	μ	κ	−	−	−	−	−	−	−
BA 7.8.55	μ	κ	−	−	−	−	−	−	−
BA 27.20.13	μ	κ	−	−	−	−	−	−	−
BA 25.24.18	μ	κ	−	−	−	−	−	−	−
BA 25.20.20	μ	κ	−	−	−	−	−	−	−
BA 29.19.43	μ	κ	−	−	−	−	−	−	−
BA 28.4.6	μ	κ	−	−	−	−	−	−	−
BA 24.11.19	μ	κ	−	−	−	−	−	−	−
BA 19.7.6	μ	κ	−	−	−	−	−	−	−
BA 28.5.33	μ	κ	−	−	−	−	−	−	−
BA 8.24.32	γ	κ	−	−	−	−	−	−	−
BA 6.10.9	μ	κ	−	−	−	−	−	−	−
BA 28.22.21	μ	κ	−	−	−	−	+	−	−
BA 30.22.6	μ	κ	−	−	−	−	−	−	−
BA 7.15.7	μ	κ	−	−	−	−	−	−	−

* Detected as described in Materials and Methods.
‡ DNP-BSA.
§ Egg lysozyme.
‖ Ribonucleoproteins.
¶ Hemagglutinin of influenza A/PR/8/34 virus.

when tested undiluted. Thus, it appears that clonal dominance to PC is the result of selective forces imposed by the antigen that act during and not before the immune response. The lack of reactivity with PC of the clones studied is consistent with the fact that none of our hybridomas express the T15 idiotype. As this marker is selectively expressed when V_HT15 combines with $V_κ22$ (8, 12), our results demonstrate that in the preimmune repertoire this $V_κ$ segment is not overrepresented among V_HT15 clones. These data therefore underlie the strength of selective forces that act during the immune response that result in the dominant expression of a single combination of V gene segments that are underrepresented in the preimmune repertoire.

Our results are apparently in discordance with those reported by Klinman and Stone (14), who observed that $T15^+$ B cells occur as a high frequency event even within the bone marrow–generative cell pool. The discrepancy between these data and ours, however, is only apparent since the results of these authors are based on the analysis of bone marrow Ig^- cells that differentiate and are expanded in vitro in the presence of PC antigens. Our clones, on the other hand, were selected exclusively on the basis of their V_H expression and independently of their antigen specificity. Taken together, our results establish that clonal dominance is dictated by the nature of the hapten, which selects, among several

TABLE II
Characteristics of C.B20 V_HT15^+ Hybridomas

Hybridoma	Isotype		Idiotypes*		Reactivity pattern‡				
	H	L	GB4.10	AB 1.2	PC	DNP	Ly	RNP	HA
C.B 30.17.20	μ	κ	−	−	−	−	−	−	−
C.B 6.13.36	μ	κ	−	−	−	−	−	−	−
C.B 13.13.11	μ	κ	−	−	−	−	−	−	−
C.B 13.19.3	μ	κ	−	−	−	−	−	−	−
C.B 14.24.6	μ	κ	−	−	−	−	−	+	−
C.B 19.6.12	μ	κ	−	−	−	−	−	−	−
C.B 19.21.35	μ	κ	−	−	−	−	−	−	−
C.B 24.21.45	μ	κ	−	−	−	−	−	−	−
C.B 14.15.10	μ	κ	−	−	−	−	−	−	−
C.B 6.14.36	μ	κ	−	−	−	−	−	−	−
C.B 9.11.7	μ	κ	−	−	−	−	−	−	−
C.B 5.7.38	μ	κ	−	−	−	−	−	−	−
C.B 18.19.26	μ	κ	−	−	−	−	+	−	−
C.B 21.13.8	γ	κ	−	−	−	−	−	−	−
C.B 11.24.8	μ	κ	−	−	−	−	−	−	−
C.B 28.24.36	μ	κ	−	−	−	−	−	−	−
C.B 24.15.14	μ	κ	−	−	−	−	−	−	−
C.B 5.11.12	μ	κ	−	−	−	−	−	−	−
C.B. 22.1.26	μ	κ	−	−	−	−	−	−	−
C.B. 17.17.29	μ	κ	−	−	−	−	−	−	−
C.B 15.3.48	μ	κ	−	−	−	−	−	−	−
C.B 10.16.7	μ	κ	−	−	−	−	−	−	−
C.B 8.16.10	μ	κ	−	−	+	−	−	−	−
C.B 8.5.17	μ	κ	−	−	−	−	−	−	−
C.B 1.20.27	μ	κ	−	−	−	−	−	−	−
C.B 24.3.8	μ	κ	−	−	−	−	−	−	−
C.B 26.7.31	μ	κ	−	−	−	+	−	−	−
C.B 8.18.46	μ	κ	−	−	−	−	−	−	−
C.B 3.19.45	μ	κ	−	−	+	−	−	−	−

* Detected as described in Materials and Methods.
‡ See footnotes to Table I.

possible candidates, only one or few of them. The mechanisms and the reasons for this selection are, at the present moment, obscure. One interesting, but still speculative possibility, is that among all potential PC-reactive clones the T15 one would be selected because it is the one that crossreacts the least with self antigens.

Our data on the other hand are compatible with those reported by Manser et al. (15), who, in a study similar to ours, analyzed the expression in the preimmune repertoire of a V_H gene segment that is dominantly expressed in the immune response to Ars in strain A mice. The conclusion of these studies was that this V gene segment can participate in encoding a large number of V region structures of which only one is used during the immune response to Ar.

IEF analysis of the L chains of our hybridoma libraries revealed that both V_HT15^a and V_HT15^b alleles are selective in terms of the V_L segments with which they combine. 10 of the 28 BALB/c hybridomas, in fact, express L chains with identical spectrotypes (Fig. 1). The two dominant L chain patterns seen among the C.B20 clones, on the other hand, are completely absent in the BALB/c collection. One possible explanation for these results is that recognition by the TC54 antibody is influenced by the L chain V region that is paired with the V_HT15. However, we find this possibility unlikely since only two hybridomas (C.B26.7.31 and C.B3.19.45) express the same light chain of the S107 protein that was used as immunogen to obtain the TC54 antibody. Our results, therefore,

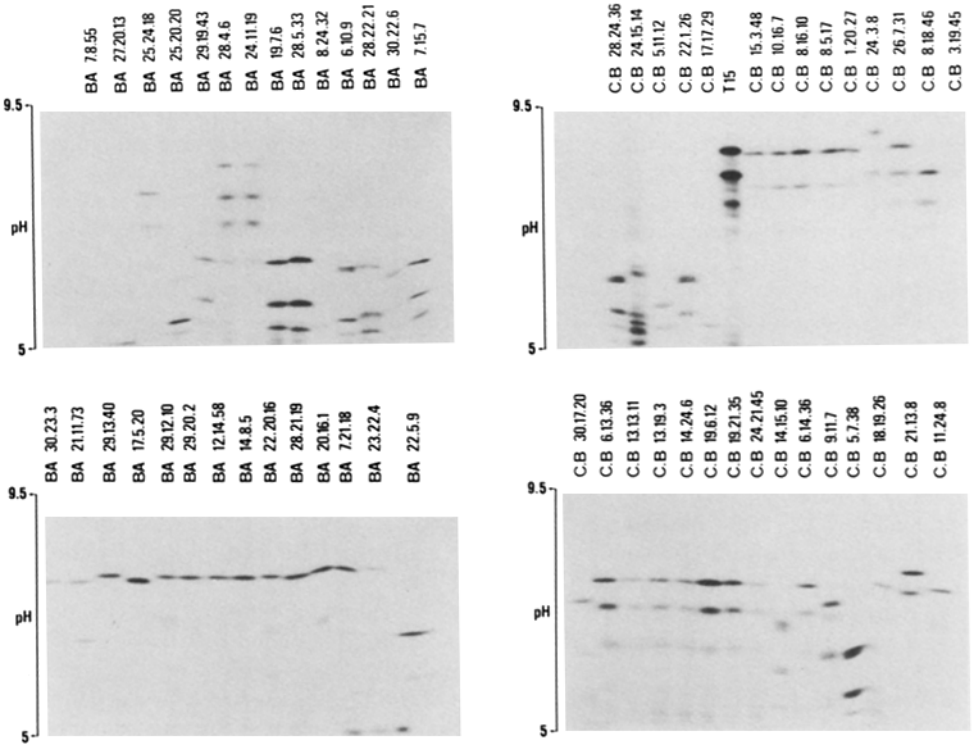

FIGURE 1. IEF patterns of L chains of 28 V_HT15^+ BALB/c (BA) and 29 V_HT15^+ C.B20 (C.B) hybridomas.

suggest that V_H–V_κ interactions are not random phenomena and that strong functional restrictions may limit, to an important degree, the extent of functional diversity.

Summary

The aim of this work was to study the cellular basis of the phenomenon of clonal dominance. To this end we analyzed two collections of BALB/c and C.B20 hybridomas that we selected on the basis of the expression of the V_HT15 gene product independently from their antigen specificity. Our study demonstrates that none of the 28 BALB/c and only 2 of the 29 C.B20 hybridomas obtained have variable regions that bind PC. We conclude therefore that the domination of the immune response to PC by particular variable regions cannot be due to the establishment of clonal dominance prior to immunization.

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