Brief Definitive Report

HUMAN MONOCLONAL ANTI-KEYHOLE LIMPET HEMOCYANIN ANTI BODY-SECRETING HYBRIDOMA PRODUCED FROM PERIPHERAL BLOOD B LYMPHOCYTES OF A KEYHOLE LIMPET HEMOCYANIN-IMMUNE INDIVIDUAL

By H. Clifford Lane, James H. Shelhamer, Howard S. Mostowski, and Anthony S. Fauci

From the Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20205

Murine hybridoma antibodies of predetermined specificity have been of great value in the study of antibody diversity, in dissecting out the roles of idiotypes and anti-idiotypes in immunoregulation, and as reagents for immunologic diagnoses (1, 2). Monoclonal human hybridoma antibodies have been produced by the fusion of human spleen (3, 4), lymph node (5), or malignant cells (6) with human or mouse myeloma cells. These methods have not gained widespread applicability for a number of reasons, including the lack of practical accessibility of these lymphoid organs and/or the undetermined specificity of the secreted monoclonal immunoglobulin (Ig). Human-human hybridomas secreting antimeasles antibodies have been produced using the peripheral blood lymphocytes of a patient with subacute sclerosing panencephalitis. However, the specific Ig produced in this manner has been of mixed genotype, containing components of both fusion partners (7). On the other hand, successful fusion has been accomplished with normal human peripheral blood lymphocytes and mouse myeloma cells. Although human antibodies have been produced in this manner, they have been of unknown specificity and were secreted in association with murine Ig and mixed mouse-human Ig (8).

We have recently developed an antigen-induced, antigen-specific in vitro system for the study of specific human B cell responses and have delineated the kinetics of the circulating antigen-specific B cell repertoire in normal human subjects following immunization with the soluble protein antigen keyhole limpet hemocyanin (KLH) (9, 10). The present study reports the successful production of a hybridoma secreting human monoclonal antibody of predetermined specificity utilizing the peripheral blood lymphocytes of one of these subjects.

Materials and Methods

Human Hybridoma Antibody Production. Human-mouse heterohybridomas were produced by a modification of the method described by Schlom et al. (5) for fusion of human lymph node cells. Unfractionated mononuclear cells were obtained by Ficoll-Hypaque gradient centrifugation of 100 ml of peripheral blood drawn from a normal volunteer 10 d after immunization with 5 mg of KLH (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.) as previously described (9). These cells were fused with an equal number of the nonsecreting BALB/c hypoxanthine-aminopterin-thymidine (HAT)-sensitive myeloma line SP-1 (kindly provided by Dr. J. Schlom, National Cancer Institute, Bethesda, Md.) utilizing polyethylene glycol-1500. Following fusion, cells were suspended at 10^7/ml in complete media, comprised of...
RPMI 1640 plus glutamine (Flow Laboratories, Inc., Rockville, Md.), 2 μg/ml amphotericin B (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.), 70 μg/ml gentamicin (Schering Corp., Kenilworth, N. J.), and 30% heat-inactivated fetal calf serum screened to support hybridoma growth (Gibco Laboratories). 100 μl of this suspension was added to each of the inner wells of a 96-well, flat-bottomed microtiter plate (Costar 3596, Costar, Data Packaging, Cambridge, Mass.). The outer wells were filled with a mixture consisting of 100 μg/ml amphotericin B and 0.5 mg/ml gentamicin. The day of fusion is denoted day 0. 24 h after fusion, 125 μl of complete media containing 4 × 10⁻⁷ M aminopterin, 1 × 10⁻⁴ M hypoxanthine, and 1.6 × 10⁻⁵ M thymidine (all from Sigma Chemical Co., St. Louis, Mo.), hereafter referred to as HAT media, was added to each well. On days 2, 3, 5, 8, 11, 14, 17, and 21, 100 μl of supernatant was removed from each microwell and 100 μl of fresh HAT media added.

On day 21, all supernatants were screened for nonspecific human IgG or IgM and for IgG or IgM directed against KLH using enzyme-linked immunosorbent assays (ELISA) as has been described previously (9, 11). Cultures secreting the desired Ig were aspirated and transferred to individual 16-mm wells of a 24-well culture plate (Costar 3524) containing 1 ml of complete media supplemented with 1 × 10⁻⁴ M hypoxanthine and 1.6 × 10⁻⁵ M thymidine (HT media). 24 h after transfer, an additional 1 ml of HT media was added to each well. Thereafter, 1 ml of supernatant was exchanged for 1 ml of fresh HT media twice weekly.

After 14 d in macroculture, wells were again screened for antibodies against KLH. Cell lines continuing to secrete specific antibody were transferred to 75 cm² tissue culture flasks containing 5 ml of complete media. Cell lines were then expanded and either frozen or used for cloning.

The cell line with the best growth characteristics secreting the highest titer-specific antibody was chosen for cloning by limiting dilution. For the first cloning, cells were cultured in complete media and plated at 100, 10, or 1 cell/well in 96-well round-bottomed microtiter plates. Positive wells, screened at 7-14 d, were transferred to 16-mm wells and then expanded into flasks as described above. They were then cloned twice at 0.5 cell/well.

To obtain purified antibody, cloned cells were grown to confluence in RPMI 1640 supplemented with 2 μg/ml amphotericin B and 70 μg/ml gentamicin, with or without 20% fetal calf serum. Cell suspensions were then centrifuged at 1,000 g, and the supernatants were decanted.

Isoelectric Focusing. Analytical isoelectric focusing was performed as previously described with a 110-ml column (8100, LKB Instruments, Stockholm, Sweden) (12). A pH gradient of 3.5-10 was constructed in a sucrose gradient (50-0% wt/vol) in 2 M urea/1% ampholytes. The sample (150 μl of affinity-purified antibody in phosphate-buffered saline at 0.5 mg/ml) was applied to the middle of the gradient. The column was run at a constant 300 V for 72 h at 4°C and eluted in 0.5 ml fractions. Eluted fractions were assayed for anti-KLH antibody using an alkaline-phosphatase ELISA with the amount of antibody present expressed as OD₄₀₀ (absorption of paranitrophenol).

Double Immunodiffusion. 20 μl of commercial antiserum (Meloy Laboratories, Springfield, Va. or N. L. Cappel Laboratories, Cochranville, Pa.) or affinity-purified hybridoma antibody was applied to each well of an Ouchterlony plate. Photographs were taken after a 48-h incubation.

Lymphocyte Blast Transformation. Cultures were performed as previously described in 96-well microtiter dishes (Limbro Chemical Co., Hamden, Conn.) with 1 × 10⁵ cells/well (13). Cultures contained 0.2 ml RPMI 1640 with 15% human A serum. Various amounts of anti-KLH hybridoma antibody, pooled human IgM myeloma (N. L. Cappel Laboratories), or pokeweed mitogen (PWM; Gibco Laboratories) were added to the cultures that were then incubated in a 5% CO₂ atmosphere at 37°C. Cultures were pulsed with 2 μCi of [³H]thymidine on day 5 and harvested 4 h later on a Titertek cell harvester (Flow Laboratories). The filter disks were placed in scintillation vials with 3 ml Aquasol scintillation fluid (New England Nuclear, Boston, Mass.) and counted in a scintillation counter (Beckman LS-350, Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.).

Results

On initial screening 102 of 240 wells were found to contain Ig (Table I). Eight of these contained antibodies directed against KLH. Six of the cell cultures secreting
antibodies specific for KLH were expanded into larger plates, and two of these six continued to produce anti-KLH antibodies at 5 wk. The faster growing line, 2F7, was chosen for cloning. The initial cloning efficiency was low (<12%), and the cells had to be plated at 100/well in order to obtain a rapidly growing antibody-producing line. Once this was achieved, however, subsequent cloning was possible at 0.5 cell/well, with a cloning efficiency of 64% in the final cloning.

The hybridoma antibody produced by the fusion of human peripheral blood lymphocytes with the nonsecreting mouse myeloma line SP-1 was a human IgMk (Fig. 1a and b), achieving concentrations in culture as high as 30 μg/ml. No mouse Ig (heavy or light chain) was detectable by double immunodiffusion. Affinity-purified antibody displayed a single peak on isoelectric focusing with a pI of 4.5, thus confirming its monoclonal nature (Fig. 2).

Addition of the monoclonal anti-KLH antibody to otherwise unstimulated cultures of peripheral blood mononuclear cells from individuals recently immunized to KLH resulted in a substantial blastogenic response. Although the mononuclear cells from nonimmunized individuals failed to proliferate in response to the monoclonal antibody 2F7, the peripheral blood lymphocytes of subjects recently immunized to KLH showed a 10-fold increase in [3H]thymidine incorporation in response to 10 μg/ml of 2F7 (Table II).

Discussion

In the present study we describe the production of a B cell hybridoma from human peripheral blood lymphocytes secreting human monoclonal antibody directed against a predetermined antigen to which an individual was recently immunized. Several factors were influential in our ability to achieve a stable heterohybridoma secreting KLH-specific antibody. It was important to take advantage of the fact that antigen-specific B cells will be contained in relatively high proportions in the circulating peripheral blood pool for only a short time after immunization with the antigen in question (10, 14). Appreciation of this restriction in the time interval when antigen-reactive B cells are available in the circulating B cell repertoire for fusion following immunization cannot be overemphasized. The transience of these antigen-specific B
Fig. 1. Analysis of 2F7 antibody by double immunodiffusion. A, goat anti-human IgM (μ-chain specific); B, goat anti-human IgG (γ-chain specific); C, goat anti-human IgA (α-chain specific); D, sheep anti-human λ; E, goat anti-human κ; F, goat anti-mouse IgM (μ-chain specific); G, rabbit anti-mouse IgG (heavy and light chain); H, rabbit anti-mouse κ. Precipitin lines between wells E and D and between wells B and C represent cross-reactivity between the immunizing antigen (human Ig) and goat or sheep Ig.

Fig. 2. Isoelectric focusing of 2F7. Procedure as described under Materials and Methods. 0.5-ml fractions were obtained and assayed for anti-KLH activity and pH. O, pH; ●, OD<sub>405</sub> (absorption of paranitrophenol in ELISA).

**Table II**

| Subject | KLH-immune | Unstimulated (background) | PWM* | 2F7‡ | IgM§ |
|---------|------------|---------------------------|------|------|------|
| 1       | Yes        | 179 ± 10†               | 9,632 ± 362 | 1,349 ± 194 | 140 ± 16 |
| 2       | Yes        | 1,369 ± 365             | 25,253 ± 637 | 9,795 ± 1012 | 1,067 ± 105 |
| 3       | No         | 345 ± 36                | 11,672 ± 1316 | 182 ± 25 | 289 ± 50 |
| 4       | No         | 218 ± 28                | 4945 ± 461 | 203 ± 17 | 202 ± 53 |
| 5       | No         | 155 ± 8                 | 10,146 ± 657 | 261 ± 62 | 163 ± 92 |

* 1:200 of stock solution.
† 10 μg/ml.
‡ Pooled human myeloma (N. L. Cappel Laboratories) 10 μg/ml.
§ Data are expressed as cpm/10⁵ cells ± SEM for triplicate cultures.
cells in the circulation had previously made detection of antigen-specific B cell
responses in human peripheral blood extremely difficult. Recent studies have high-
lighted the critical nature of the kinetics of appearance and disappearance of these
cells in the circulation in relation to immunization (10, 14). In addition, technical
considerations, such as initial cloning at a high cell density to increase the probability
of obtaining a rapidly dividing, stable, high-titer antibody-secreting cell line, and the
use of 30% fetal calf serum along with round-bottomed plates for the limiting dilution
cloning, were instrumental in the successful establishment of the hybridoma.

Several investigators have attempted to increase their yield of antigen-specific
hybridomas by culturing with doses of antigen sufficient to cause substantial lympho-
cyte blastogenesis before fusion (4). In view of the recent data that blastogenic doses
of antigen may, in fact, suppress rather than enhance specific B cell responses (10), we
have chosen not to take this approach. However, the ability of submitogenic doses of
antigen (those doses which induce specific antibody production in vitro) to enrich for
antigen-specific B cell hybridomas is currently under investigation.

Although the cell line producing the monoclonal antibody is a heterohybridoma,
its producing pure human Ig. While several of the KLH-specific lines generated in
the fusion that produced 2F7 were unstable and ceased secreting Ig after several weeks,
the 2F7 line has remained stable and in continuous culture for the past 10 mo. In this
regard, others have also noted stable as well as unstable antibody-producing lines
following heterohybridization (8). Until stable and reliably fusible, HAT-sensitive
human cell lines become available, heterohybridization should be a perfectly accept-
able and widely applicable means of obtaining human monoclonal antibodies of
predefined specificity. The previously reported antigen-specific human hybridomas
secreting monoclonal antibodies have used lymph node or spleen as the fusion partner.
The accessibility of peripheral blood, as opposed to lymphoid organ B cells, enhances
the general applicability of the method described here.

The anti-KLH hybridoma antibody was found to be mitogenic for the peripheral
blood lymphocytes of individuals who had been immunized to KLH but not for the
lymphocytes of nonimmune individuals. The significance of this finding is not clear
at present, but may represent stimulation of a subset of immune lymphocytes bearing
anti-idiotypic receptors as has been described in other systems (15).

The ability to produce human monoclonal antibodies of predefined specificity from
normal human peripheral blood opens major possibilities for basic as well as applied
clinical research. Studies of antibody diversity, the roles of idiotypes and anti-idiotypes
in immunoregulation, as well as the therapeutic uses of specific antibody should be
greatly facilitated by the simplicity and clearcut feasibility of this approach.

Summary

A human IgMk monoclonal antibody, 2F7, of predetermined specificity, has been
produced by the fusion of human peripheral blood lymphocytes with the nonsecreting
mouse myeloma line SP-1. The heterohybridoma has remained stable for over 8 mo
with culture supernatants containing up to 30 μg/ml of specific IgM. The antibody
has been shown to be capable of inducing a blastogenic response in the absence of
antigen in the peripheral blood lymphocytes of normal subjects immune to the
antigen.

The ability to choose an antigen, immunize, a human subject to that antigen, and
then use the peripheral blood lymphocytes from that subject to produce antigen-specific human monoclonal antibodies should be of great value in a wide variety of investigative, diagnostic, and therapeutic endeavors.

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