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Immunology

Production of antibodies against influenza virus by somatic cell hybrids between mouse myeloma and primed spleen cells

(cell fusion/anti-hemagglutinin antibodies/malignancy)

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ABSTRACT **We have produced somatic cell hybrids between mouse myeloma cells and spleen cells derived from a BALB/c mouse immunized with purified influenza virus. The hybrid cells were found to produce large amounts of antibodies specific for the hemagglutinin of the virus and were able to induce tumor formation when injected into BALB/c mice.**

Monoclonal antibodies specific for antigenic determinants of viruses such as influenza (1) or parainfluenza I (2) have been produced *in vitro* in the splenic fragment system. Until now, however, the production of antibody by spleen foci in culture has been declining after 30–40 days and ceasing altogether after 90 days. In our observation, any attempts to transform cells of the splenic fragments by either simian virus 40 or Abelson murine leukemia virus ended in failure. The selection *in vitro* of continuous cell lines that produce antibodies of given specificities would greatly facilitate antigenic analysis of viral determinants. The availability of such antibody might also be useful in immunotherapy.

In the present study we have succeeded in the selection of somatic cell hybrids between influenza virus-primed mouse spleen cells and mouse myeloma cells. These hybrids can be maintained indefinitely in culture and continue to produce anti-influenza antibodies.

MATERIALS AND METHODS

Preparation of Spleen Cells for Fusion. Two to three months after being primed by an intraperitoneal (i.p.) injection of 1250 hemagglutinating (HA) units of purified influenza virus [A/PR/8/34(HONI), referred to as PR8], BALB/c mice received an intravenous booster dose of 100 HA units of homologous virus. The mice were sacrificed 2 days later and a spleen cell suspension was prepared (3). Erythrocytes were lysed by incubation for 15 min at 4° in NH_4Cl (0.83%). The resulting cell suspension was washed by one centrifugation (800 × *g*) through heat-inactivated calf serum and one centrifugation in protein-free medium [RPMI 1640, buffered with 7.5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), pH 7.2].

Myeloma Cells. BALB/c myeloma cells (P3 × 63 Ag8) derived from the MOPC-21 line (4) and deficient in hypoxanthine phosphoribosyltransferase (HPRT) (EC 2.4.2.8) (5, 6), were obtained through the kindness of Cesar Milstein of the Medical Research Council Laboratory of Molecular Biology, University Postgraduate Medical School, Cambridge, England. They were maintained in Eagle's minimal essential medium (MEM) containing 10% fetal calf and 10% horse serum.

Abbreviations: i.p., intraperitoneal; HA, hemagglutinin; MEM, minimal essential medium; HAT, hypoxanthine/aminopterin/thymidine; RIA, radioimmunoassay; PBS, phosphate-buffered saline; Ig, immunoglobulin.

Production of Hybrids. Ten million P3 × 63 Ag8 cells were mixed with 10^8 spleen cells obtained from PR8-immunized BALB/c mice. The cell mixture was centrifuged at 300 × *g* and the cells were resuspended for fusion in a 50% solution (wt/vol) of polyethylene glycol (PEG) 1000 (7) which was diluted first with MEM only and then with MEM and serum, according to established fusion procedures (8). Cells were seeded in five 75-cm^2 Falcon flasks in hypoxanthine/aminopterin/thymidine (HAT) selective medium (9). The cultures were incubated at 37° in an atmosphere of 95% air/5% CO_2, and every 7–10 days one-half to one-third of the culture medium was partially replaced by fresh HAT medium.

Karyologic Analysis. Karyologic analysis of the P3 × 63 Ag8 cells, the hybrid cells, which were called HK-PEG-1, and the clones was performed after trypsin–Giemsa banding staining according to a modification of the method described by Seabright (10, 11).

Radioimmunoassay (RIA). The quantitation and analysis of anti-influenza antibodies were performed by RIA as described previously (1, 3) except that the immunoadsorbent in the form of virus bound to the wells of soft plastic plates (Cooke Engineering) was prepared as described by Rosenthal *et al.* (12). The anti-$F(ab')_2$ serum was produced by immunizing rabbits with $F(ab')_2$ obtained by pepsin digestion of murine IgG; the class-specific antisera were purchased from Meloy Laboratories, Inc., Springfield, VA. All antisera were purified on the corresponding mouse-Ig-bromoacetyl-cellulose and were iodinated by the chloramine-T method (13).

Functional Assays for Anti-Influenza Antibodies. Hemagglutination inhibition was performed as described by Fazekas de St. Groth and Webster (14). Neuraminidase inhibition was done according to the procedure recommended by the World Health Organization (15).

Analysis of the Immunoglobin Produced by the Hybrid Cells. HK-PEG-1 cells were injected i.p. into 20 (BALB/c × NZB)F_1 mice (obtained from the Institute for Cancer Research, Fox Chase, PA) primed with 0.5 ml of pristane (2,6,10,14-tetramethylpentadecane, Aldrich Chemical Co.) 2 weeks prior to receiving the tumor cells. The ascitic fluids collected from these mice were pooled, and the pool was diluted with phosphate-buffered saline (PBS). An equal volume of saturated ammonium sulfate was added at 4°, and the precipitated protein was dissolved in PBS and dialyzed against 0.01 M Tris·HCl buffer at pH 8.0. The precipitate formed after overnight dialysis was redissolved in PBS and reprecipitated by dialysis against 0.01 M Tris buffer at pH 8.0. The supernatant from the original dialysate was absorbed on a DEAE-cellulose (DE-52, Whatman) column equilibrated with 0.01 M Tris buffer, pH 8.0, and eluted using a linear NaCl gradient (0.05–0.15 M NaCl in 0.01 M Tris, pH 8.0).

The paraproteins in sera or ascitic fluids of mice bearing ei-

Table 1. Number of chromosomes in parental and hybrid cells

Cells	Average no. of chromosomes per cell
P3 × 63 Ag8	63*
BALB/c spleen cells	40
HK-PEG-1	92*

* Includes two metacentric marker chromosomes.

ther P3 × 63 Ag8 or HK-PEG-1 tumors and the fractions isolated from the ascitic fluids (see previous paragraph) were characterized as to heavy chain class by Herbert C. Morse (National Institutes of Health). Samples were analyzed by immunoelectrophoresis using Ig-class-specific goat antisera.

RESULTS

Production of Hybrids. Ten to fifteen days after the incubation of cultures produced by the fusion of spleen cells of PR8-immunized mice with P3 × 63 Ag8 cells, cell growth was observed in one flask. These cells, referred to as HK-PEG-1, were propagated continuously in HAT medium and were cloned in microplates (Linbro) at limiting dilution. Four other flasks, none of which showed cell growth, were discarded 3 weeks after fusion.

Karyological Analysis. As shown in Table 1, the P3 × 63 Ag8 cells contained an average of 63 chromosomes and the BALB/c spleen cells, 40 chromosomes. Thus, the 92 chromosomes in the HK-PEG-1 cells represented approximately the sum of chromosomes of the two parental cells. The karyology of the hybrid clone HK-PEG-1 and of subclones was monitored for 4 months with no significant change in the karyotype.

Immunoglobulins Produced by Parental and Hybrid Cultures. Culture fluids of HK-PEG-1 and P3 × 63 Ag8 cells were analyzed for the presence of immunoglobulins (Ig) reacting in the RIA with PR8. P3 × 63 Ag8 is known to secrete IgG1,κ (6). As shown in Table 2, P3 × 63 Ag8 culture fluids did not contain Ig with anti-PR8 reactivity. In contrast, large quantities of IgG anti-PR8 antibodies were produced by HK-PEG-1. The anti-PR8 antibodies produced by the hybrid cells were specific for the hemagglutinin (HA) of the PR8. This is evident from the functional assays (Table 3) in which the antibodies (roughly 40 μg/ml) exhibited a high hemagglutination inhibition but no detectable neuraminidase inhibition.

Clonal Origin of the Hybrid Cells. The clonal origin of HK-PEG-1 was tested by two independent criteria. First, the antibody was tested in the RIA for its crossreactivity against various influenza viruses* known to be antigenically related to PR8 and was found to be specific for a determinant of the HA of PR8. However, 20–25% of splenic PR8-primed precursor B cells exhibit this strain-specific anti-HA (PR8) reactivity (1). Taken alone, therefore, the antibody reactivity is a rather weak criterion for determining the monoclonality of HK-PEG-1. Second, in order to exclude the possibility that cells producing antiviral antibody constituted only a small fraction of the hybrid cell population, culture fluids derived from 12 clones of the HK-PEG-1 hybrid line were assayed for the presence of anti-PR8 antibody and its specificity. The results, shown in Table 4, indicate that 11 of the 12 clones produced anti-PR8 antibody. Furthermore (not shown in Table 4), these antibodies were specific for a determinant of the HA of the PR8 virus.

Table 2. Class of anti-PR8 antibody produced by HK-PEG-1 cultures

Culture fluid assayed in RIA*	cpm observed in RIA with ^{125}I-labeled antisera			
	F(ab')$_2$	IgM	IgA	IgG
P3 × 63 Ag8	44 ± 24†	N.D.	N.D.	185
HK-PEG-1	4255 ± 158†	204	26	8335

N.D., not determined.

* Medium was from cultures with similar cell concentrations. Replicate samples of 15 μl (P3 × 63 Ag8) or 7.5 μl (HK-PEG-1) of culture fluid were analyzed.

† Mean ± SEM of six determinations. Other entries: mean of RIA done in duplicate.

Table 3. Activity of antibody produced by HK-PEG-1 in functional assays against PR8 virus

Antibody	Titer ($\log_{10}$) against PR8	
	Hemagglutination inhibition	Neuraminidase inhibition
Secreted *in vitro* by HK-PEG-1 and concentrated*	2.98	<1.00

* Fifty microliters of HK-PEG-1 culture medium was precipitated with ammonium sulfate at 4° at 42% saturation. Precipitate was dialyzed against PBS and concentrated by ultrafiltration to 0.6 ml. This final sample contained, as determined by RIA, 47% of the antiviral antibody present in the starting volume of culture medium and thus represents, roughly, a 40-fold concentration of antiviral antibodies.

Tumorigenicity of the Hybrid Cells. Adult BALB/c mice were injected subcutaneously in the abdominal wall with 10^7 cells of either HK-PEG-1 or P3 × 63 Ag8.

Ten to twelve days after implantation (Table 5), tumors developed at the site of inoculation in 5/5 mice injected with the HK-PEG-1 cells and in 3/5 mice injected with the parental P3 × 63 Ag8 cells. In the second experiment, 4/5 mice developed tumors after inoculation with hybrid cells and 3/5 after inoculation with P3 × 63 Ag8 cells. After i.p. injection of 10^7 cells per mouse (Exp. 3, Table 5), tumors were found to grow as masses in the peritoneal cavity. In order to produce ascitic fluid, it was necessary to inject the mice with tumor cells resuspended in complete Freund's adjuvant. In Exp. 4, pristane-primed mice developed ascites after i.p. inoculation of cells of three HK-PEG-1 clones (see Table 5).

Presence of Anti-Influenza Antibodies in Sera and Ascitic Fluids Obtained from Mice Injected with HK-PEG-1 Cells. Sera and ascitic fluids were obtained from tumor-bearing mice at various intervals after injection, and the concentration of anti-PR8 antibody was determined by means of RIA (3) (Table 6). Sera of mice injected subcutaneously with HK-PEG-1 cells (Table 6, Exps. 1 and 2) contained anti-PR8 antibodies at a concentration of 1–3 mg/ml. Anti-PR8 antibodies were also found in the ascitic fluid of mice injected i.p. (Exp. 4) with hybrid cells, although the concentration of antibodies was 3- to 4-fold lower than in the serum.

Electrophoresis of serum obtained from mice bearing HK-PEG-1 tumors revealed the presence of three main Ig popula-

* WSE, BH, Mel, Hickcox, Bel, and Weiss representing the A0 (H0N1) subtype, and Cam, FM1, and Denver representing the A1 (H1N1) subtype.

Table 4. Anti-PR8 antibody produced by clones of HK-PEG-1 cultures

Culture fluid from HK-PEG-1 clone no.	Concentration of anti-PR8 HA antibody,* μg/ml
2	2
3	1.2
5	2.4
6	3.4
7	4.7
9	6.9
10	4.7
11	5.8
12	<0.02
13	2.8
14a	2.7
14b	2.6

* Quantitation based on RIA done with ^{125}I-labeled anti-F(ab')$_2$.

tions: one corresponding to the parental (P3 × 63 Ag8) IgG1; one characteristic of IgG3; and a third intermediate between the other two. In order to determine with which class of immunoglobulins the antiviral antibody was associated, ascitic fluid was collected from 20 pristane-primed mice and the immunoglobulins were separated as described in *Materials and Methods*.

Following precipitation of the IgG3 by dialysis against 0.01 M Tris buffer (see *Materials and Methods*), the remaining supernatant was found, by immunoelectrophoresis, to contain IgG1 and IgG3 heavy chain determinants (Fig. 1, sample 4). This material was chromatographed on DEAE-cellulose (Fig. 2). The concentration of anti-PR8 antibody was determined in the RIA using ^{125}I-labeled anti-F(ab')$_2$, and the specific anti-PR8 activity was computed as the ratio anti-PR8 (mg/ml)/Ig (mg/ml), the latter estimate being based on the A_{280} measurement and assuming an absorptivity (1%, 1 cm) for mouse Ig of 14.0. The specific anti-PR8 activity determined in the low-salt precipitate was roughly three times higher than at the peak of the anti-PR8 activity of the DEAE-cellulose chromatography (0.04 in fraction 37). Analysis of the samples in the

Table 5. Tumorigenicity for mice of the hybrid and parental myeloma cells

Exp.	Route of inoculation	Inoculum	Ratio of mice developing tumors: Subcutaneous	Ascitic*
1	s.c.	HK-PEG-1	5/5	—
		P3 × 63 Ag8	3/5	—
2	s.c.	HK-PEG-1	4/5	—
		P3 × 63 Ag8	3/5	—
3	i.p.	HK-PEG-1 in CFA	—	3/3
		HK-PEG-1	—	0/3
4†	i.p.	HK-PEG-1 cl 6	—	4/4
		HK-PEG-1 cl 7	—	4/4
		HK-PEG-1 cl 12	—	4/4

s.c., subcutaneous; i.p., intraperitoneal; CFA, complete Freund's adjuvant; cl, clone.

* A — indicates that no ascitic tumor had developed 30 days after inoculation.

† Pristane-primed mice.

Table 6. Anti-PR8 antibodies in sera and ascitic fluids obtained from mice injected with 10^7 HK-PEG-1 cells per mouse

Exp.	Days after injection when material obtained	Material	Concentration of antiviral antibody,* mg/ml
1	28	Serum	1.35
		Serum	1.1
		Serum	2.3
2	19	Serum	0.9
		Serum	1.6
		Serum†	1.2
		Ascites†	0.5
4	15	Ascites cl 6	0.5
		Ascites cl 7	0.450
		Ascites cl 12	<0.002

cl, clone.

* Estimated by RIA with ^{125}I-labeled anti-F(ab')$_2$.

† From the same mouse.

RIA with ^{125}I-anti-IgG1 indicated that only approximately 15% of the anti-PR8 antibodies in the low-salt precipitate expressed G1 determinants. In contrast, ^{125}I-anti-IgG1 was as effective as ^{125}I-anti-F(ab')$_2$ in quantitating the anti-PR8 antibodies in the various fractions of the DEAE-cellulose chromatography (see Fig. 2). In addition, immunoelectrophoretic analysis of fraction 37 (leading peak fraction of anti-PR8 activity) demonstrated the presence of heavy chain determinants of both IgG1 and IgG3.

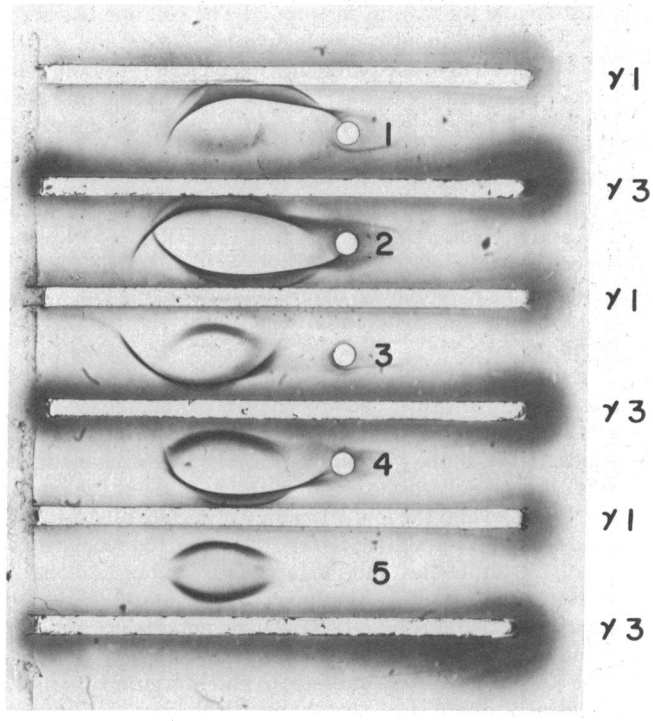

FIG. 1. Immunoelectrophoresis was kindly performed by Herbert C. Morse, National Institutes of Health. Troughs: goat antisera specific for heavy chain determinants of either mouse IgG1 (γ1) or IgG3 (γ3). Wells: ascitic fluid produced by P3 × 63 Ag8 (well 1) or HK-PEG-1 (well 2). Low-salt precipitate (0.01 M Tris) of HK-PEG-1 (well 3), supernatant after low-salt precipitation of HK-PEG-1 (well 4), and fraction 37 of DEAE-cellulose chromatography of supernatant (well 5) (see FIG. 2).

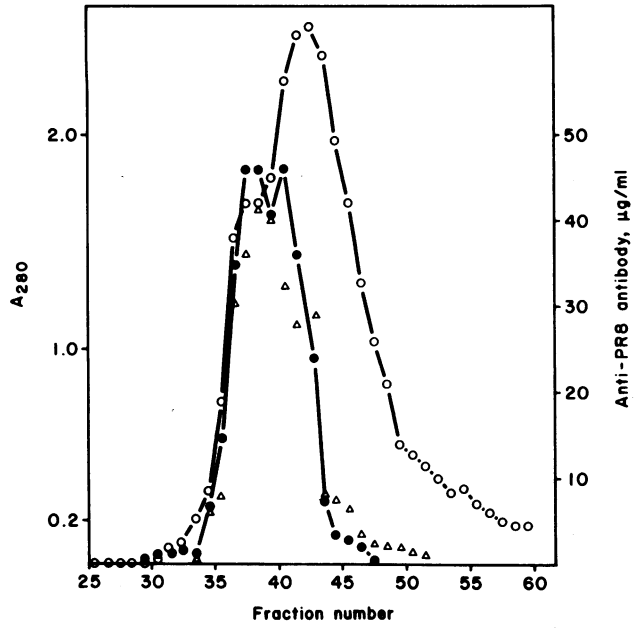

FIG. 2. Material and chromatographic conditions are described in *Materials and Methods*. Symbols: absorbance at 280 nm (○); concentration of anti-PR8 antibody determined in RIA with ^{125}I-labeled anti-F(ab')$_2$ (●) or anti-IgG1 (△).

DISCUSSION

This study confirms that the cell line described represents a hybrid culture that displays characteristics of both the normal spleen and the myeloma parental cells and is probably derived from a single fusion event. The hybrid nature of the cells seems unquestionable for several reasons: (*i*) The cell line has been grown for several months in selective HAT medium, which inhibits the growth of the parental P3 × 63 Ag8 myeloma cells but not of the normal spleen cells which, alone, would probably not survive for more than 4–5 weeks *in vitro*. Spontaneous revertants of P3 × 63 Ag8 to HAT resistance apparently do occur but only rarely (6). (*ii*) The number of chromosomes in the hybrid cells is close to the sum of chromosomes in normal mouse and myeloma parent cells. (*iii*) The HK-PEG-1 hybrid produced not only IgG1, which is also secreted by P3 × 63 Ag8, but also IgG3 and, probably, hybrid molecules. (*iv*) The hybrid produced antibodies with antiviral activity, whereas P3 × 63 Ag8 did not.

The derivation of the cell line from a single fusion event is more difficult to prove because hybrid cultures may be genetically unstable, allowing variants to arise within a few generations (6, 16). In the present analysis, the following evidence points convincingly to the origin of the hybrid culture from a single fusion event: the low efficiency of successful growth of hybrid cells (one out of five flasks), the restricted reactivity of the antiviral antibodies, and the fact that 11 out of 12 clones of the original mass culture produced antiviral antibodies with identical specificities. Clone 12, which did not produce anti-PR8 antibodies, may very likely represent a spontaneous variant.

The studies of Milstein *et al.* (5, 6) and Margulies *et al.* (16) have clearly established that hybrid cells may continue to produce Ig chains characteristic of both parental cells. Immunoelectrophoretic data indicate that the HK-PEG-1 hybrid produced not only the parental P3 × 63 Ag8 IgG1 myeloma protein, but at least two additional Ig species, one representing IgG3 and the other exhibiting characteristics of both IgG1 and IgG3. Such findings indicate that IgG3 is the other parental Ig and, consequently, exhibits the anti-PR8 reactivity. This supposition is further supported by the fact that the specific anti-PR8 activity was highest in the IgG3-enriched fraction. On the other hand, after low-salt precipitation of IgG3 the supernatant still contained considerable quantities of anti-PR8 antibodies. However, because these antibodies were detected in the RIA using either class-specific anti-IgG1 antibodies or anti-F(ab')$_2$ antibodies with the same efficiency (Fig. 2) and because the parental (P3 × 63 Ag8) IgG1 myeloma protein did not exhibit anti-PR8 activity, it seems that the antibodies in the supernatant represent hybrid molecules of the two parental immunoglobulins.

This study indicates that it is possible to select *in vitro* hybrid cells that produce large quantities of monoclonal antibodies against specific viruses and their antigenic determinants. These studies also indicate that hybrid cells between mouse myeloma and normal spleen cells behave as the malignant parent and that no suppression of malignancy is observed in this type of hybrid.

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