Development of Monoclonal Antibodies to
Human Fibroblast DNA Polymerase α

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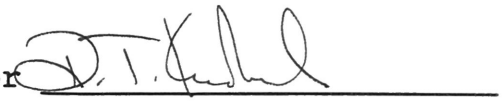
Honors Director 

ABSTRACT

A regimen for the production of a monoclonal antibody that can distinguish between adult-expressed and fetal-expressed DNA polymerase α has been developed, but has not yet been completed. Hybrid BALB/c x C57BL/6 mice were immunized 3 x intraperitoneally with polymerase over a 2 month interval. During the first immunization, ace-mannan was also introduced as an immunomodulator to boost antibody production and secretion by plasma cells. Two weeks after the last injection, the spleen cells were fused separately with two non-immunoglobulin secreting murine myeloma cell lines, Sp2/0-Ag14 and P3X63/Ag8.653. Four weeks later, the supernatant from two wells with hybridoma colonies were tested for antigen specificity by enzyme-linked immunosorbent assay (ELISA). Due to a lack of readily available antigen and an inaccurate standard antibody titration curve in the ELISA checkerboard titration, the cells were frozen at -70°C so that a more reliable ELISA system could be established without permitting cell overgrowth. Once ELISA systems for the two isozymes of DNA polymerase α are operational, the plates of hybridomas will be thawed, tested and cloned by limiting dilution until a monoclonal antibody is developed that can distinguish between the fetal and adult isozymes.

INTRODUCTION

DNA polymerase α (DNA nucleotidyltransferase, E.C. 2.7.7.7), a vital eukaryotic nuclear enzyme, accounts for the addition of 70-90% of the deoxyribonucleotidyl monophosphates in the 3' to 5' direction of the template strand during DNA replication and DNA excision repair (Fry and Loeb, 1986). In humans, DNA polymerase α occurs in two age-related forms, adult and fetal (A_1 and A_2, respectively) (Sylvia et al., 1988; Busbee et al., 1988). The occurrence of A_1 in normal human fibroblasts increases from 0% to 94% of the total cytoplasmic polymerase α as the age of the donor increases from a 16-week fetus to 66 years, while A_2 decreases correspondingly (Busbee et al., 1988, CRC). The two isozymes exist as tetramers with three of the peptide subunits, including the 156 to 200 kDa catalytic subunit, being identical, and the fourth differing to an unknown degree. Although the two forms can be isolated from one another, to do so requires the employment of very expensive techniques that involve up to six chromatographic procedures. The development of a monoclonal antibody that can differentiate one form from the other is the purpose of this project.

EXPERIMENTAL PROCEDURES

MATERIALS

Six male BALB/c X C57BL/6 F_1 mice (Jackson Laboratory, Bar Harbor, Me.) were immunized with DNA polymerase α that was isolated from the 2RA SV40-transformed human fibroblast cell line (American Type Culture Collection, Rockville, Md.). The enzyme was purified using DEAE-cellulose, immunoaffinity and DNA-

cellulose procedures and was injected along with ace-mannan (Carrington Laboratories, Irving, Tx.) in the initial mouse immunization. The two non-secreting mouse myeloma cell lines, Sp2/0-Ag14 (Ozato and Sachs, 1981) and P3X63-Ag8.653 (Kearney et al., 1979), were also from the American Type Culture Collection.

All cell cultures were grown in Iscove's modified Dulbecco's medium (IMDM) (Gibco Laboratories, Grand Island, N.Y.) with penicillin, streptomycin, gentamycin (Sigma Chemical Company, St. Louis, Mo.) and fungizone (Grand Island Biological Company Grand Island, N.Y.). The fetal bovine serum (HyClone Laboratories, Logan, Utah) supplemented the IMDM during myeloma maintenance and after hybridization. The HD3-100 polyamide nylon fiber mesh used in teasing the splenocytes was from Tetko, Inc. (Elmsford, N.Y.). For the cell fusion, Carbowax polyethylene glycol m.w. 4000 (PEG 4000) from Fischer Scientific (Fair Lawn, N.J.) was used. To assure that unfused myeloma cells would die, hypoxanthine-aminopterin-thymidine (HAT) and HT from Sigma supplemented the media.

The cell cultures were grown, on Falcon 24-well polystyrene plates (Becton Dickinson and Co., Lincoln Park, N.J.), and the enzyme-linked immunosorbent assays (ELISAs) were performed on Corning 96-well ELISA plates (Corning, N.Y.). The Tween-20 detergent used in washing the plates was from Sigma (St. Louis, Mo.). The PBS/10% BSA diluent/blocking solution, as well as the ABTS (2,2'-azino-di(3-ethyl-benzothiazolin-sulphonate))/H_2O_2 chromagen solution were from Kirkegaard and Perry Laboratories (Gaithersburg, Md.). The peroxidase conjugated goat anti-mouse

antibody was from Cooper Biochemical (Malvern, Pa.).

METHODS

Preparation of splenocytes

Six male BALB/c X C57BL/6 mice were given one 0.25 ml intraperitoneal injection of 50% DNA polymerase α (530 μg/ml) and 50% ace-mannan. The following two immunizations, given at two to three week intervals, consisted solely of the immunogen. Two weeks after the last immunization, three of the mice were killed by cervical dislocation, and their spleens removed. (The spleens of the remaining three mice were prepared later for fusion with the second myeloma cell line, P3XP63/Ag8.653.) The tissue was finely cut and mashed using a sterile, disposable #10 scalpel, and the clumps of cells were dispelled by pipetting up and down with a trochar syringe. With the syringe plunger, the plasma cells from the spleen were teased from the solid tissue through a polyamide nylon mesh. The cell suspension was transferred to a 50 ml centrifuge tube, centrifuged at low speed, washed in IMDM and kept on ice until the hybridization.

Cell fusion

The myeloma cells from two 150 cm^2 flasks were harvested, centrifuged and resuspended in a 50 ml centrifuge tube along with the washed spleen cells. The spleen cells and the myeloma cells were mixed at a ratio of 10:1 (respectively) in a 50 ml centrifuge tube, packed by low speed centrifugation and the supernatant was decanted. 0.5 ml of warm (37°C) 50% PEG 4000 (Lane et al., 1984) in serum-free IMDM was added dropwise over

one minute and with slight agitation to the cells. 10 ml of serum-free IMDM was then added slowly over five minutes, and the cells were left to stand for ten minutes. Finally, the cells were resuspended in 40 ml of IMDM-HAT with 10%FBS, and the centrifuge tube was washed with another 10 ml of IMDM-HAT to retrieve remaining cells, bringing the total volume of selective medium to 50 ml. The cell mixture was aliquoted out into 24-well plates (1 ml/well). The plates were incubated at 37°C in a humid atmosphere of 7.5% CO_2 in air.

Hybridoma maintenance

The hybridomas were observed daily with an inverted microscope to monitor cell growth and possible contamination. Every second or third day for fourteen days, 0.5 ml of spent medium was removed and replaced with 0.5 ml of fresh IMDM-HAT. The HAT medium was eliminated after the two weeks, and the colonies were fed (50:50) IMDM-HT with 10% FBS for an additional two weeks. Four weeks after fusion, the growing colonies were fed IMDM/10% FBS. Wells that appeared to have hybridoma colonies were tested for the presence of antigen-specific antibody by ELISA (see below). Four of the six 24-well plates were frozen at -70°C after the cells were fed a freezing medium of IMDM/20% FBS with 5% dimethyl sulfoxide (DMSO). One of the plates had yeast contamination in nearly every well and was discarded. The other had yeast or bacterial contamination in only some of the wells. The cells from those uncontaminated wells were salvaged and grown in 25 cm^2 tissue culture flasks until enough cells could be frozen.

ELISA

Checkerboard titration.

The purified sample of DNA polymerase α was shown to have a total protein concentration of 373 μg/ml, using the Bio-Rad Protein Assay. Six dilutions of the enzyme were made in 0.05 M sodium carbonate/bicarbonate solution pH 9.6 to give the following concentrations: 300, 250, 200, 150, 100 and 50 μg/ml. Two plates were coated with 100 μl/well of the enzyme at 4°C overnight, and the dilutions went from left to right (columns 2 through 7). Unadsorbed antigen was expelled by washing the plates once with phosphate-buffered saline containing 0.05% Tween-20 (PBST) (Gardas and Lewartowska, 1988), using a Dynatech Dynawasher II. To block any open sites on the plate, a commercial solution of PBS/10% BSA was diluted 10x and added (100 μl/well). After about 2 hours of blocking at room temperature, the wells were washed twice with PBST. A 3 μg/ml standard antibody sample diluted in PBS/1% BSA was added (100 μl/well), incubated at room temperature for 30 minutes and washed three times. The second antibody, a peroxidase-conjugated goat anti-mouse antibody, was diluted in PBS/1% BSA to give the following concentrations: 5.00, 2.50, 1.25 and 0.625 μg/ml. To the first two rows, the second antibody was added at a concentration of 5 μg/ml; to the next two rows, at 2.5 μg/ml; and so on until the eight rows were filled (all at 100 μl/well). The plates were allowed to incubate for 45 minutes at room temperature and were washed five times. An ABTS/H_2O_2 chromagen solution was added (150 μl/well), and the absorbances were read at 490 nm after incubation at 37°C for 30 minutes, using the MR 600 Dynatech

Microplate Reader.

For each concentration of second antibody, the absorbances were plotted as a function of antigen concentration. Once optimal levels of enzyme and second antibody concentrations were obtained, they were applied to a third ELISA plate to determine the optimal concentration of the first antibody. The plate was coated overnight at 4°C with the antigen at a concentration of 150 μg/ml, and was washed, blocked and washed again. The standard antibody was diluted 10-fold in PBS/1%BSA and was added to the plate with dilutions going from left to right. After incubating and washing, the second antibody was added at a concentration of 5 μg/ml. The plate was incubated, and the chromagen solution added. The absorbances were read at 490 nm and plotted on a graph to set a limit for positive and negative results.

Cloning by limiting dilution

Because the ELISA optimization did not give adequate results and too many variables were present in the system (see ELISA results, below), the cells were frozen to prevent possible overgrowth by non-immunoglobulin secreting hybridomas. Once a reliable ELISA system has been established, the 24-well plates of hybridomas will be thawed in a 37°C incubator. To assure again that the cells are hybridomas, they will be fed IMDM with hypoxanthine and thymidine (no aminopterin). The supernatants of each well will be tested by ELISA for the presence of antigen-specific antibodies. The cells from those positive wells will be grown in a 25 cm^2 tissue culture flask until cell division can be

detected. The dividing cells will be transferred to a 15 ml centrifuge tube, packed by low speed centrifugation and resuspended in IMDM/10% FBS to give a cell count of 10^2 to 10^3 cells/ml. The cell suspension will be diluted two-fold in tubes and then aliquoted out (300 µl/well) into the appropriate wells of a 96-well tissue culture plate with dilutions going from left to right. After four days, the supernatants will be tested for activity. The cells from active wells will be diluted again, if necessary, or will be transferred to regular medium for further culture expansion for analysis or freezing. Analysis will include testing antibody activity against polymerases A_1 and A_2 and assaying enzyme activity after antibody binding.

OBSERVATIONS

The hybridomas produced from the Sp2 cell line seemed to be more susceptible to the HAT medium than those from the P3X63 cell line. Most of the unfused Sp2 cells had died within a period of 24 hours in HAT, whereas most of the P3X63 myelomas were still viable after 40 hours. The P3X63 cells were dividing so rapidly after the fusion that an acidic condition was noted after 24 hours of growing in HAT medium. The phenol red indicator in the IMDM had turned a bright yellow, indicating that the pH dropped to as low as 6.5 from a normal pH of 7.2.

Bacteria contaminated two wells of the Sp2 hybridomas despite the presence of penicillin, streptomycin and gentamycin in the medium, and yeast survived the 2x fungizone. This resulted in a loss of 62% of the Sp2 wells.

RESULTS

The ELISA checkerboard titration demonstrated the optimal antigen concentration to be 150 μg/ml and the optimal conjugated antibody concentration to be 5 μg/ml (Table 1, Figure 1). Titration of the first antibody showed a peak at a 1000-fold dilution, but showed somewhat smaller, but still significant, peaks at 10^7- and 10^9-fold dilutions (Table 2, Figure 3). At these dilutions the antibody was in 0.3 nanogram and 3.0 pecogram amounts, respectively. The titration curve was expected to level off at a certain absorbance above which results would be designated positive and below which would be designated negative (Figure 4). The data are shown below.

Table 1. ELISA Checkerboard Titration: Determination of Antigen and Second Antibody Concentrations. Absorbance (490 nm)

	[Antigen] (μg/ml)					
[2° Ab] (μg/ml)	300	250	200	150	100	50
5.000	0.0320	0.0415	0.0425	0.0485	0.0375	0.0350
2.500	0.0220	0.0255	0.0395	0.0305	0.0330	0.0300
1.250	0.0195	0.0270	0.0310	0.0220	0.0225	0.0310
0.625	0.0225	0.0230	0.0255	0.0225	0.0225	0.0280

Figure 1. ELISA Checkerboard Titration:
[Antigen] and [Second Antibody] Determination

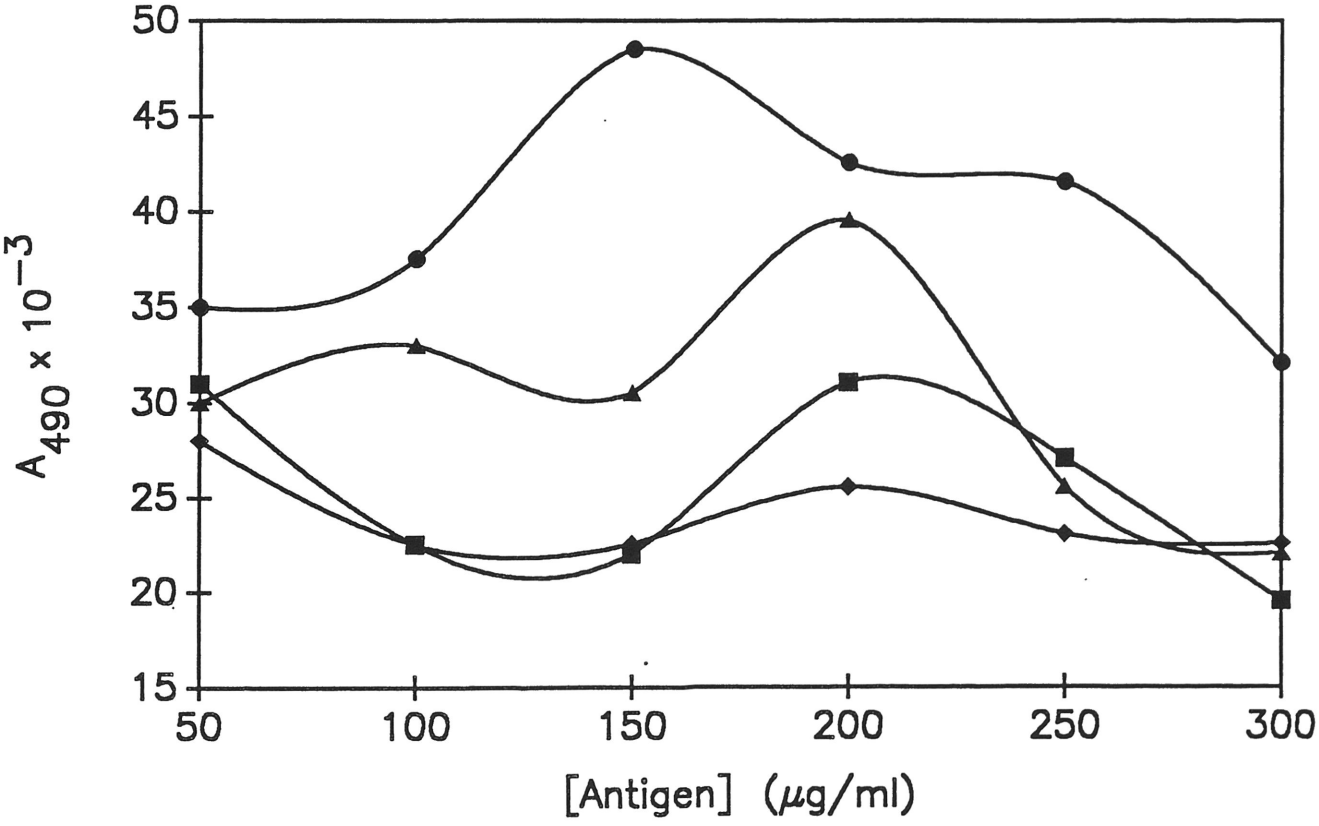


Figure 2. Ideal Checkerboard Titration Curve for
[Antigen] and [Second Antibody] Determination

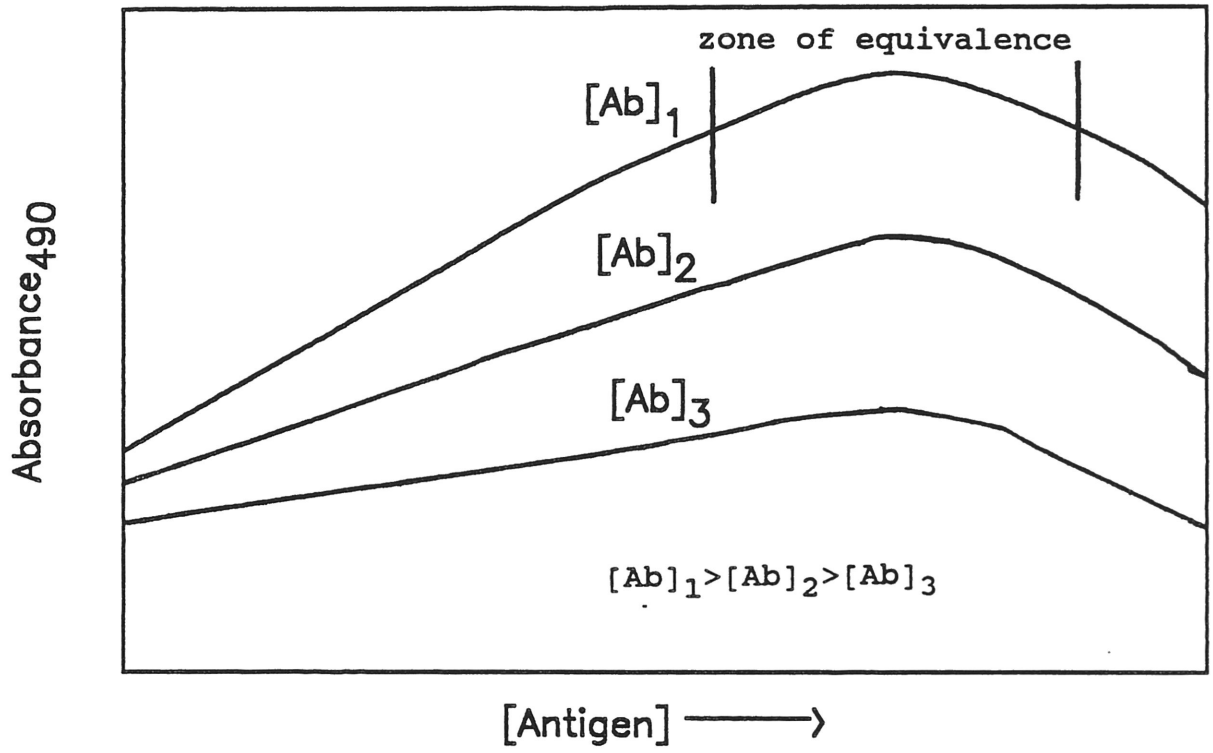


Figure 3. ELISA Checkerboard Titration:
[First Antibody] Determination

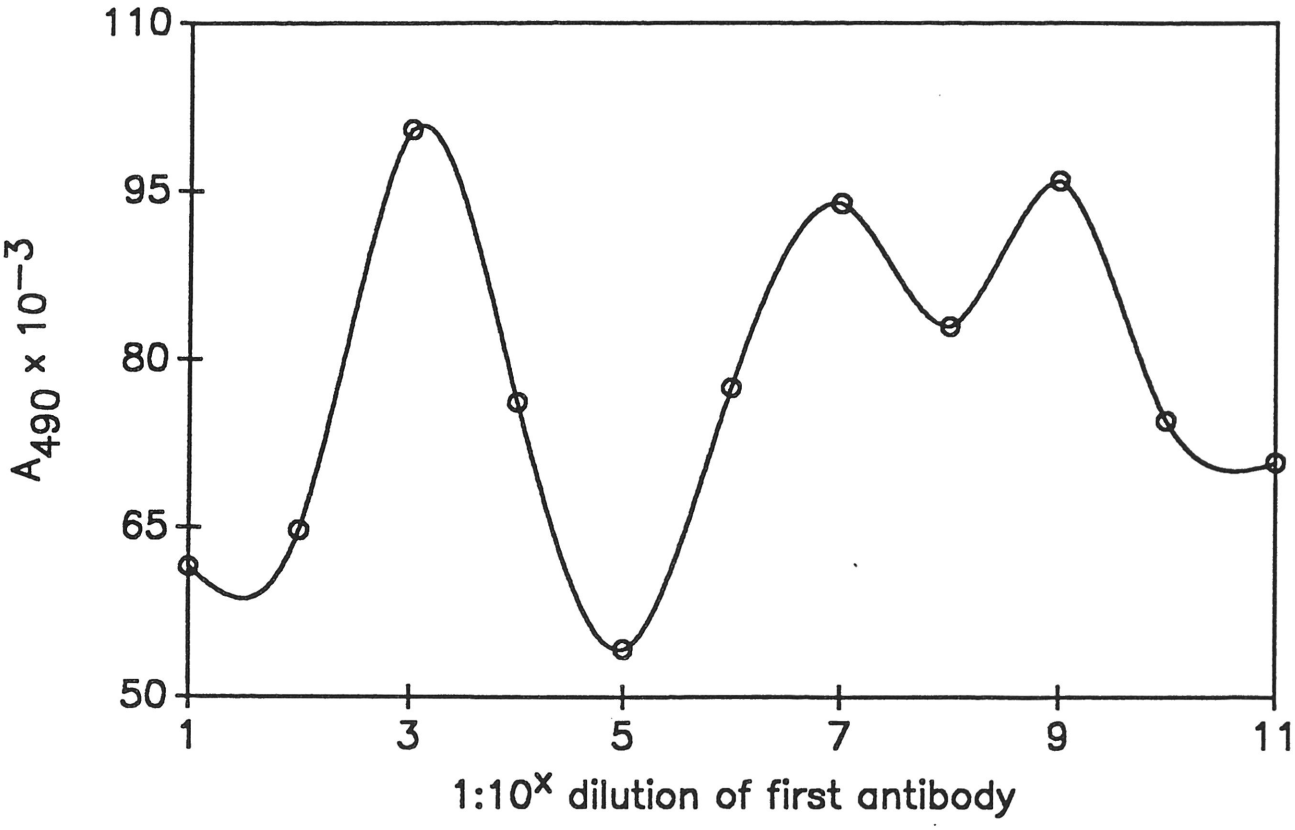


Figure 4. Ideal Checkerboard Titration for
[Standard Antibody] Determination

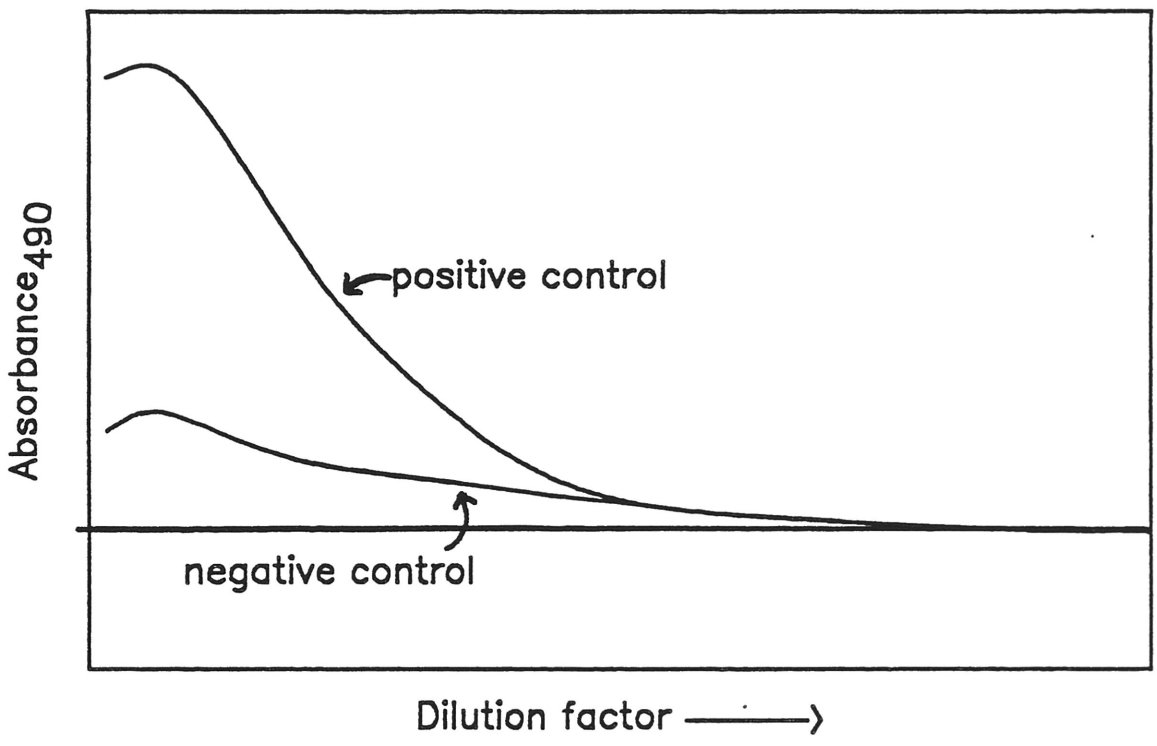


Table 2. ELISA Checkerboard Titration: Determination of Standard Antibody Concentration.

dilution factor	Absorbance (490 nm)				avg.
10^1	0.080	0.074	0.042	0.050	0.06150
10^2	0.074	0.049	0.076	0.060	0.06475
10^3	0.082	0.174	0.079	0.067	0.10050
10^4	0.077	0.105	0.055	0.068	0.07625
10^5	0.059	0.041	0.055	0.062	0.05425
10^6	0.067	0.079	0.066	0.098	0.07750
10^7	0.119	0.060	0.125	0.072	0.09400
10^8	0.061	0.083	0.078	0.110	0.08300
10^9	0.077	0.146	0.065	--	0.09600
10^{10}	0.074	0.085	0.083	0.056	0.07450
10^{11}	0.057	0.078	0.067	0.061	0.07075

DISCUSSION

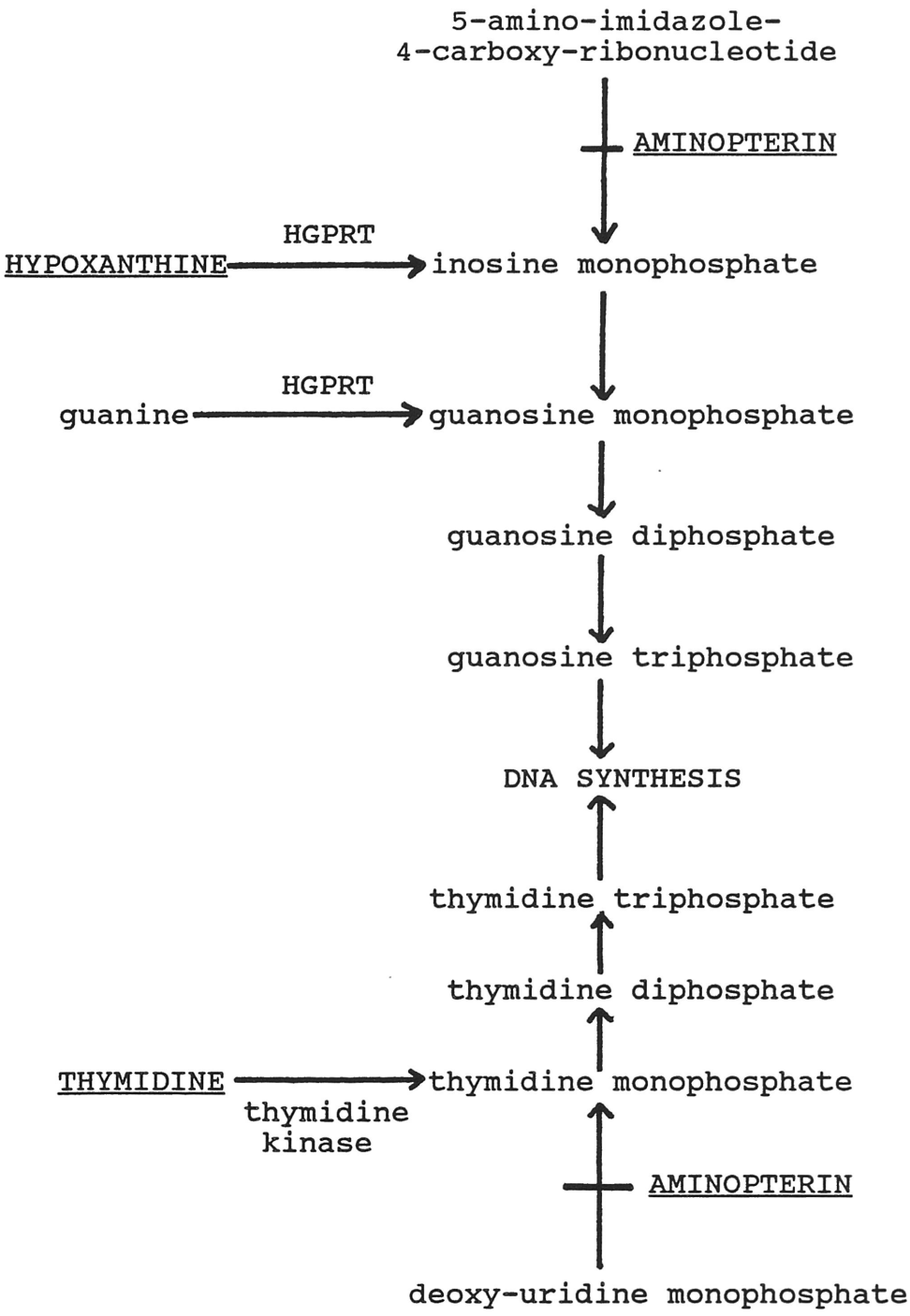
Basis for HAT selection

The myeloma cell lines used for cell fusion have a genetic marker that allows only hybridized myeloma cells to survive in selective medium. Hypoxanthine, aminopterin and thymidine (HAT) in the medium serve this purpose. Aminopterin, a dihydrofolic acid analog, blocks the main biosynthesis pathways of inosine monophosphate (IMP, a precursor for guanosine monophosphate, GMP) and thymidine monophosphate (TMP) necessary for DNA synthesis. Normal myelomas can continue to synthesize these nucleotides by utilizing the salvage pathways catalyzed by the enzymes hypoxanthine guanine phosphoribosyl transferase

(HGPRT) and thymidine kinase, provided that hypoxanthine and thymidine are present as the respective substrates. The HAT selection is actually a half-selection in which the Sp2 and P3X63 myeloma cell lines are $HGPRT^-$ mutants, but have the gene that codes for thymidine kinase necessary to catalyze TMP synthesis. Although hypoxanthine is supplied in the medium, these mutant cells can not utilize it to synthesize IMP in the presence of aminopterin and die. Myeloma cells that have fused with splenocytes gain the HGPRT gene of the other parent cell. Since the splenocytes require special conditions for their growth that are not met with the HAT medium, they eventually die in the tissue culture or during transfer.

After fusion of the splenocytes with the P3X63 cell line, an acidic condition (pH 6.5) was noted after 24 hours, that was not observed with the Sp2 myelomas. Once the medium was replaced with IMDM/10% FBS with newly reconstituted HAT, the unfused cells began to die out. One possible explanation for this condition was that the aminopterin of the seven-day old reconstituted HAT had lost its inhibitory activity towards dihydrofolate reductase, allowing the myelomas to synthesize IMP from 5-amino-imidazole-4-carboxy-ribonucleotide.

Figure 5. Basis for HAT Selection



ELISA

The enzyme-linked immunosorbent assay is widely used to assay for antigen specificity of antibodies because of its

advantages over earlier radioimmunoassays. Preceding radioimmunoassay radiolabeled proteins had to be handled with expertise, the reagents would lose activity when stored for a long period of time, and the equipment and materials were expensive.

One of the simplest forms of ELISA is the indirect or sandwich assay. The antigen is adsorbed onto a polystyrene plate, probably as a result of hydrophobic, nonpolar interactions between the protein structures and the plastic matrix (Engvall, 1980). After the antigen is adsorbed, the excess is washed off, and available plastic sites are blocked with another protein, such as bovine serum albumin. Some of the adsorbed proteins, however, are lost during washing because 1) they are not covalently bound to the solid phase, 2) the proteins may denature or 3) the capacity for adsorption of the plastic is limited (Engvall, 1980). In order to continually block newly-opened sites that might have occurred after washing, all solutions are diluted with a PBS/1% BSA diluent/blocking solution. The antibody being tested is then added to the well. If it is specific to a site of the antigen that is not bound to the plate, an antibody-antigen interaction results. To spectrally determine this type of binding, a peroxidase-conjugated second antibody is introduced. In this experiment, the second antibody used was an anti-mouse immunoglobulin that bound to the F_c region of mouse immunoglobulin G. A green colorimetric reaction is obtained when the oxidation of the chromagen ABTS is coupled to hydrogen peroxide cleavage by the conjugated peroxidase of the second

antibody. A lack of color indicates that the second antibody did not bind to mouse immunoglobulin, therefore the first antibody was not antigen-specific.

The checkerboard titration is performed to determine the optimal concentrations of antigen and first and second antibodies. In the first assay, two unknowns are optimized as the amount of the antigen bound to the plate decreases from left to right, while the concentration of the second antibody decreases from top to bottom. As Figure 2 shows, the amount of precipitation is a function of the antigen concentration and is plotted for each concentration of second antibody. The zone of equivalence is determined by the peak absorbance given by particular antigen and second antibody concentrations. On either side of the zone of equivalence is the zone of excess: to the right, the zone of excess antigen; and to the left, the zone of excess antibody. After obtaining optimal amounts of the antigen and the second antibody, the third unknown must be optimized. A second assay is performed using the two concentrations determined in the first titration; however, this time the concentration of the first antibody is varied. Once the second half of the checkerboard titration is plotted out on a graph, an absorbance value can be assigned above which a result is considered positive, and below which a result is considered negative (Figure 4).

One setback encountered in the development of a monoclonal antibody was that the ELISA system had not been established before the myelomas and the splenocytes had been hybridized. Consequently, the hybridomas were frozen until further ELISA

standardization could be performed.

There were a number of variables that affected the reliability of the ELISA system presented, such as purity of the antigen and known activity of the antibody being used as the standard. The DNA polymerase α that was used to immunize the mice was highly purified; however, the surplus volume of immunogen was not sufficient to standardize the immunoassay. Instead, a much less pure, though readily available, antigen that had only been chromatographed through a DEAE-cellulose column served my purpose in simply practicing the ELISA techniques. One consequence of using impure antigen for the ELISA is the possibility that peroxidase was present in the sample. As a result, the peroxidase would have bound to the plate and would have reacted catalyzed the hydrogen peroxide cleavage, giving a false positive. The results of the ELISA optimization are based on the assumption that peroxidase impurities were not present to give false positives. The activity of the first antibody used to standardize the ELISA system was also assumed to be positive, since it had been derived from A_2 hybridomas, though these hybridomas had not yet been tested.

Another factor that was equally likely to give imprecise checkerboard titration curves was technique. Although the method of washing the plates was automated with the Dynawasher II, the dilutions were done manually, leaving the potential for deviation in pipetting techniques, especially on the microliter scale. This is one explanation for obtaining such an irregular titration curve for the determination of the first antibody concentration.

Because the titration curve did not level off as expected, no line of absorbance could be intrapolated to differentiate between a positive result and a negative result.

Once additional antigen is isolated from transformed human fibroblast cultures and assayed, and once the known anti-DNA polymerase α antibodies are obtained from SJK-132-20 hybridomas (American Type Culture Collection CRL 1640), a more standardized ELISA system can be constructed. The hybridomas will then be thawed, cloned by limiting dilution and assayed until a monoclonal antibody is developed that can distinguish between the two age-related forms of DNA polymerase α.

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