Elimination of Bacteriophages from Tissue Culture Serum By Affinity Chromatography

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Use of immunoabsorbents to remove bacteriophages from tissue culture serum was investigated. Immune globulins from rabbit antiserum prepared against ϕ V-1 phage were immobilized by covalent linkage to activated porous silica glass derivatives of *p*-aminoarylamine and alkylamine and to Sepharose-4B. Chromatographic columns of each material were used to filter samples of a fetal bovine serum into which had been introduced 8.1×10^3 plaque-forming units of the phage per ml. Efficiency of removal was determined by plaque assays of ϕ V-1 phage recovered in the effluent fluids. Activated but uncoupled matrices nonspecifically removed from 49 to 59% of the phages introduced into the experimental serum. A reduction of 35 to 37% in phage content occurred in the serum after filtration through columns coupled to nonantibody protein. With specific immune globulins attached, the Sepharose-4B matrix reduced the concentration of phage in the serum below a detectable quantity. No apparent alterations occurred in the growth-promoting property of serum filtered through the Sepharose-4B immunoabsorbent as measured by cloning efficiency of BHK-21, WI-38, and FRhL-2 cells. These experiments serve as a model system for use of immunoabsorbents for selective removal of bacteriophages and perhaps other extraneous microbial agents from tissue culture serum.

Bacteriophages are frequently found as contaminants in bovine serum used as a component of tissue culture media (3, 9, 10, 13). The persistence of bacteriophages once introduced into cell culture systems has been demonstrated (12). This may be one possible explanation for the presence of phages isolated from occasional lots of live viral vaccines for human use prepared in cell cultures (10, 14). Although the biological consequences of phage-contaminated vaccines for man are at present unknown, the elimination of bacteriophages from tissue culture systems is of concern to both vaccine producers and to laboratory investigators who use cell cultures in their research.

This communication describes a laboratory model system which demonstrates the usefulness of affinity chromatographic techniques (8) for eliminating phages from tissue culture serum.

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MATERIALS AND METHODS

Preparation of antisera. Antibodies to the large plaque-forming phage (ϕ V-1), previously isolated

from live measles virus vaccine (14), were produced in six rabbits by a total of four weekly footpad injections. Each rabbit received 1.0 ml of a 10^6 phage suspension in saline combined with an equal volume of complete Freund adjuvant. Antisera from bloods collected 1 week after the last injection were pooled. The immune globulin fraction was separated from the antisera by precipitation at 35% saturation with ammonium sulfate at 4 C. The precipitated globulin was resuspended in and dialyzed against phosphate-buffered saline, pH 7.2.

Preparation of immunoabsorbents. The immunoabsorbents were prepared by covalent attachment of the immune globulin to porous glass and to the agarose gel, Sepharose-4B (Pharmacia, Fine Chemicals, Sweden).

The alkyl- and arylamine derivatives of controlled pore glass (supplied by Corning Glass Works, Corning, N.Y.) were activated as previously described (18). Summarily, the arylamine derivative was diazotized and coupled to the globulin by azo linkage, and the alkylamine was coupled through a Schiff's-base reaction using glutaraldehyde (18).

The Sepharose-4B was activated by cyanogen bromide and coupled as previously described (7, 15).

To 5.0 g of each activated carrier was added 6 ml of the immune globulin solution containing 32 mg of protein per ml. Coupling was allowed to continue for 2 h at pH 8.0 and room temperature, followed by washing in phosphate-buffered saline until no protein was detectable in the wash fluids by trichloroacetic acid precipitation. The same volume and proce-

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dures were used for coupling nonantibody protein (15 mg of normal rabbit serum albumin per ml) to activated carriers.

Removal of virus by immunoadsorption. To test the efficacy of the immunoadsorbents, small columns were prepared from each of the coupled matrices and used to filter 7-ml samples of a fetal bovine serum seeded with 8.1×10^3 plaque-forming units of ϕ V-1 phage per ml. Eagle minimum essential medium was used to chase and to dilute the serum to a final concentration of 10%.

In addition, other lots of commercially available fetal bovine serum previously found to contain known concentrations of endogenous bacteriophages were filtered through the Sepharose-4B immunoadsorbent column as specified in the particular experiment.

Recovery of phage adsorbed to columns. Elution of the adsorbed phage from the immunoadsorbents was accomplished by removing the support materials and washing them in 10 ml of glycine-buffered saline, pH 2.5. Data from these experiments are expressed as percentage of phage originally adsorbed.

Determining efficacy of phage removal. The effluent and wash fluids were tested for phage content by triplicate plaque assays according to standard methods previously described with the *Escherichia coli* strain C-3000 host system (3). The number of plaque-forming units per milliliter found in the recovered fluids was multiplied by the dilution factor of the column.

RESULTS

The antiserum was found to have a K value of 2,146 when incubated with the specific immunogen for 10 min at 37 C according to procedures recommended by Adams (1). Characteristics of the three immunoadsorbent columns are shown in Table 1. The immune globulin solution that was coupled to the columns retained 98% or more of the antibody activity found in the original rabbit antisera and at a 10-fold dilution decreased the titer of ϕ V-1 phage by more than three logs. However, the antibody covalently coupled to the three matrices did vary in quantity which must be considered when examining the data. In all other respects, the three columns were similar.

Data in Table 2 compare the efficiency of the three immunoadsorbents for the removal of the bacteriophage. Activated but uncoupled derivatives nonspecifically reduced the concentration of phage in the seeded serum by about 50%. However, when the carriers were coupled to rabbit serum albumin, only about one-third of the phages were removed. Increased effectiveness and performance of the arylamine and Sepharose-4B columns became apparent, however, when coupled to the specific antibody. Under these conditions, 98 and 100%, respectively, of the phage content in the serum were

TABLE 1. Characteristics of the immunoadsorbent columns

Characteristic	Column		
	Alkylamine	Arylamine	Sepharose
Column dimensions (mm)			
Ht	35.0	35.0	35.0
Diam	23.5	23.5	23.5
5-g bed vol (ml)	15.0	15.0	15.0
Flow rate (ml/min)	1.0	1.0	1.0
Void vol (ml)	5.0	5.0	7.0
Concn of protein (mg/ml) ^a	1.9	2.2	16.7
Load vol (ml)	7.0	7.0	7.0
Vol recovered (ml)	21.0	28.0	21.0

^a The immune globulin solution coupled to the columns diluted 10-fold decreased the titer of ϕ V-1 phage by more than three logs.

TABLE 2. Removal of bacteriophage from laboratory-contaminated serum by affinity column filtration^a

Type of activated matrix	Titer in effluent (PFU/ml $\times 10^3$) ^b			Dissociated from immunoadsorbent complex by acid ^c
	Uncoupled to protein	Coupled to non-specific protein	Coupled to specific antibody	
Alkylamine	3.3 (59) ^d	5.3 (35)	2.7 (67)	22
Arylamine	3.3 (59)	5.3 (35)	0.2 (98)	90
Sepharose-4B	4.1 (49)	5.1 (37)	0 (100)	0

^a Seven-milliliter aliquots of serum containing 8.1×10^3 plaque-forming units (PFU) of ϕ V-1 phage per ml.

^b Volume corrected for column dilution.

^c Percentage of the calculated PFUs theoretically adsorbed and subsequently released in glycine-buffered saline, pH 2.5.

^d Percentage of PFUs removed.

removed by filtration. Adsorptions with the immunoadsorbents used as a slurry (batch-type adsorption) rather than as a bed in columns gave similar results. Phages specifically adsorbed to the porous glass-derived immunoadsorbents but not those on Sepharose-4B could be partially dissociated from the immunoadsorbent complex in glycine-buffered saline. The dissociated phages retained their ability to infect and lyse the *E. coli* host bacteria.

Four successive filtrations of a lot of ϕ V-1 phage-contaminated serum with a combined total of 1.7×10^8 plaque-forming units failed to saturate completely a 4-ml bed Sepharose-4B column. The concentration of phage in the fourth serum eluate was reduced by 88%. In all, less than 10% of the theoretical binding capacity of the column was exhausted. (The theoretical binding capacity was calculated by the fol-

lowing assumptions and equation: [i] 0.02% retained activity of protein after attachment to Sepharose-4B matrix; [ii] 160,000 as the average molecular weight of the antibody molecule; and [iii] moles of antibody X%-retained activity/average gram molecular weight of antibody $\times$ Avogadro's number $\cong$ number of available sites. Ideally, the amount of antibody in the immunoglobulin used could be determined more precisely by the quantitative precipitin assay if milligram quantities of the phage were available.)

It was of interest to determine whether an immunoadsorbent prepared with a specific phage antibody (ϕ V-1) would be useful in removing other extraneous phages normally found in commercial lots of fetal bovine serum. Several lots of serum previously found to contain bacteriophages which produced different size plaques on lawns of *E. coli* were filtered through a Sepharose-4B column. Results are shown in Table 3. Of the seven lots of serum filtered, the phage concentration was reduced in three lots, with the greatest reduction occurring in titer of the large plaque-type phage. The bacteriophages not removed, presumably, are a different type from those against which the antibody was elicited, although no further attempts to type these viruses have been made.

Results obtained from cloning efficiency studies indicated that serum recovered from the Sepharose-4B immunoadsorbent could be successfully used for plating BHK-21, WI-38, and FRhL-2 cells (Fig. 1).

DISCUSSION

Previous studies conducted in this laboratory demonstrated that certain physical or chemical treatments of bacteriophage-contaminated serum failed to destroy the phages without rendering the serum useless for cultivating cells in vitro (unpublished observations). Results obtained from the experiments reported here indi-

FIG. 1. Effect of affinity chromatographic filtration on growth-promoting property of fetal bovine serum for three tissue culture cell lines. Symbols (matrix used for filtration): ■, None; ▨, Sepharose; ▤, arylamine; ▧, alkylamine.

cate that immunoadsorption offers a promising approach to the problem of phage contamination in tissue culture serum. The Sepharose-4B immunoadsorbent matrix did effectively remove the ϕ V-1 phages from serum without an apparent deleterious effect on its growth-promoting properties. Failure of the glass-derived materials in this regard may have been due to either their inherent properties or, more likely, to our experimental conditions which failed to couple adequate amounts of antibody protein to the glass matrices. Compared to the Sepharose-4B matrix with 250.5 mg of antibody protein coupled, the alkylamine and arylamine glass matrices with protein concentrations of 28.5 and 33.0 mg, respectively, probably contained unsaturated reactive sites. Under these conditions, the unsaturated reactive sites would likely remove essential proteins from tissue culture serum during filtration. This assumption appears to be substantiated by results of the cloning efficiency study wherein serum filtered through the alkylamine column provided the poorest growth condition for cells. However, further efforts to improve the performance of the glass-derived materials were not explored in the present study.

Bioselective adsorption on inert matrices has proved to be useful in separating various classes of biological substances (4, 5, 8, 16, 18). Although the present study as a model system applied the principle of biospecific adsorption by immobilized antibodies for removal of a specific bacteriophage, the technique should lend itself as well for the selective removal of other microbial agents such as mammalian viruses and mycoplasmas that have been found in tissue culture serum (2, 6, 11, 17). Because the adsorption reaction was highly specific, it is unlikely that affinity chromatography, per se, would afford a practical means to eliminate all the different types of undersirable agents that may be present in contaminated tissue culture

TABLE 3. Removal of extraneous bacteriophages naturally found in tissue culture serum

Serum lot	Titer of phage (PFU/ml) ^a				Bacteriophages removed by column (%)
	Pre-filtered		Post-filtered		
	Large	Small	Large	Small	
A		8.7 × 10 ²		8.7 × 10 ²	0
B		5.7 × 10 ²		5.7 × 10 ²	0
C		5.8 × 10 ²		5.8 × 10 ²	0
D		2.4 × 10 ²		2.4 × 10 ²	0
E		2.3 × 10 ²		0.2 × 10 ²	91
F		1.7 × 10 ⁶		0.9 × 10 ⁶	49
G	1.6 × 10 ³	5.4 × 10 ²	0.01 × 10 ³	0.4 × 10 ²	93-99

^a PFU, Plaque-forming units.

serum. However, the usefulness of polyvalent antiserum in this regard is not precluded.

LITERATURE CITED

1. Adams, M. H. 1959. Bacteriophages. Interscience Publishers, Inc., New York.
2. Barile, M. F., and J. Kern. 1971. Isolation of mycoplasma arginini from commercial bovine sera and its implication in contamination of cell cultures. *Proc. Soc. Exp. Biol. Med.* 138:432-437.
3. Chu, F. C., J. B. Johnson, H. C. Orr, P. G. Probst, and J. C. Petricciani. 1973. Bacterial virus contamination of fetal bovine sera. *In Vitro* 9:31-34.
4. Cuatrecasas, P. 1971. Selective adsorbents based on biochemical specificity, p. 79-109. *In* G. R. Stark (ed.), *Biochemical aspects of reactions on solid supports*. Academic Press Inc., New York.
5. Cuatrecasas, P., and C. R. Anfinsen. 1971. Affinity chromatography. *Annu. Rev. Biochem.* 40:259-277.
6. Fong, C. K. Y., P. A. Gross, G. D. Hsiung, and N. S. Swack. 1975. Use of electron microscopy for detection of viral and other microbial contaminants in bovine sera. *J. Clin. Microbiol.* 1:219-224.
7. Gelb, W. G. 1973. Affinity chromatography for separation of biological materials, p. 61-65. *In* American laboratory, Oct. 1973. International Scientific Communications, Inc., Green Farms, Conn.
8. Lowe, C. R., and P. D. G. Dean. 1974. Affinity chromatography. John Wiley & Sons, Inc., New York.
9. Merrill, C. R., T. B. Friedman, A. F. Attallah, M. R. Geier, K. Krell, and R. Yarkin. 1972. Isolation of bacteriophages from commercial sera. *In Vitro* 8:91-93.
10. Moody, E. E. M., M. Trousdale, G. H. Jorgensen, and A. Shelokov. 1975. Bacteriophages and endotoxin in licensed live virus vaccines. *J. Infect. Dis.* 131:588-591.
11. Molander, C. W., A. J. Kniazeff, C. W. Boone, A. Paley, and D. T. Imagawa. 1971. Isolation and characterization of viruses from fetal calf serum. *In Vitro* 7:168-173.
12. Orr, H. C., P. G. Probst, D. C. Littlejohn, F. C. Chu, and J. C. Petricciani. 1974. Persistence of bacteriophages in experimentally infected cell cultures. *Appl. Microbiol.* 27:1105-1107.
13. Orr, H. C., K. H. Sibinovic, P. G. Probst, H. D. Hochstein, and D. C. Littlejohn. 1975. Bacteriological activity in unfiltered calf serum collected for tissue culture use. *In Vitro* 11:230-233.
14. Petricciani, J. C., F. C. Chu, and J. B. Johnson. 1973. Bacteriophages in live virus vaccines. *Proc. Soc. Exp. Biol. Med.* 144:789-792.
15. Porath, J. 1974. General methods and coupling procedures, p. 13-30. *In* W. B. Jakoby and M. Wilchek (ed.), *Methods in enzymology*, vol. 34B. Academic Press Inc., New York.
16. Scouten, W. H. 1974. Affinity chromatography: bioselective adsorption on inert matrices, p. 26-39. *In* American Laboratory, Aug. 1974. International Scientific Communications, Inc., Green Farms, Conn.
17. Swack, N. S., C. K. Y. Fong, G. D. Hsiung, and P. A. Gross. 1975. Methods for the detection of viruses in bovine serum. *J. Clin. Microbiol.* 1:212-218.
18. Weetall, H. H., and A. M. Filbert. 1974. Porous glass for affinity chromatography applications, p. 59-72. *In* W. B. Jakoby and M. Wilchek (ed.), *Methods in enzymology*, vol. 34B. Academic Press Inc., New York.