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Use of chromatography for the preparation of homologous tetanus antitoxin

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ABSTRACT

To obtain immunoglobulin of selected action with a higher titre, techniques for isolating antibodies from a donor's normal immunoglobulin by means of affinity chromatography were used. To obtain an affinity sorbent, tetanus toxoid was used as a ligand. Using an immunosorbent based on the purified ligand, tetanus antibodies were prepared with a specific activity of 65 IU/mg. According to gel-permeation chromatography, the tetanus antibodies obtained were represented mainly by IgG monomer (>90%). The anticomplementary activity of the preparations obtained, being at least 11 mg of protein per 2CH50, does not rule out the possibility of intravenus administration of tetanus antibodies.

INTRODUCTION

In spite of considerable advances in tetanus control, primarily due to planned immunization, the problem of treating this disease still remains urgent. When the pathogen enters the blood, even individuals vaccinated with antitoxin at a concentration below 0.01 IU/ml (IU = International Unit) are unprotected against the disease. In this case it is advisable to use antitetanus heterologous or homologous immunoglobulin, having high preventive and therapeutic effects. Antitetanus homologous immunoglobulin has high preventive and therapeutic effects and in a number of countries it is used for protection against disease and has almost completely replaced the use of horse antitetanus serum. This is associated with the possible anaphylactogenous property of the given preparation, although in the U.S.S.R. it is common practice to use DIAFERM-3 [mainly F(ab')2, and with rapid elimination of heterologous F(ab')2 fragments of tetanus antibodies from the body in 10–12 h] [1]. However, severe cases of tetanus are still treated with heterologous preparation, because so far no antitetanus immunoglobulin suitable for intravenous administration has been produced on a large scale. This is due to several reasons, mainly the lack of raw materials arising, in turn, from a limited donor supply and inadequacy of immunization programmes. This problem might be solved by a drastic increase in the number of donors or by their hyperimmunization [2]. However, as the data presented by Wellhoner [1] show, even a single immunization may cause adverse effects in a number of cases, ranging from urticaria to the Arthus phenomenon. On the other hand, only 1.5% of routine donor plasma has a level of tetanus antibodies of 10 IU/ml and can, therefore,

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be fractionated by Con's method, which is capable of a 10-fold concentration of antibodies in the final product [3].

An alternative method for obtaining γ-globulin with a selective action is therefore of interest, in particular a technique for isolating antibodies from raw materials with a high titre by means of affinity chromatography, especially as precedents are known (a similar preparation is produced by Merier, France) [4]. Affinity chromatography makes it possible to extract from a mixture of different immunoglobulins only those molecules which exert antitetanus activity, even if they are present at trace levels. For this purpose tetanus toxoid, which is one of the components of the biospecific pair, is fixed on a matrix as the so-called "ligand", and with its aid its complementary antitetanus immunoglobulin is extracted. After removal of foreign molecules, the eluent composition is altered in such a way as to destroy the antigen–antibody complex and the released antibodies are eluted from the carrier.

In spite of the fact that polysaccharide-type matrices (Sephadex, cellulose, agarose) are widely used for fractionating biological macromolecules, they show no promise in industrial application owing to a number of physico-chemical properties. To solve these problems, after preliminary experiments with various types of macroporous silica (MPS), MPS-2000 with the required properties for chromatography (incompressibility, resistance to breakage, good hydrophilicity, tolerance to a high sample throughput in the chromatographic process and ability for sterilization) was adopted as a matrix [5]. A hydrophilic spacer having an active epoxy group at the end was used as a glass modifier both for elimination of non-specific adsorption of molecules on the surface of the matrix and for linking tetanus toxoid.

In this paper we describe the results obtained in the extraction of tetanus antibody from commercial normal γ-globulin preparations by column affinity chromatographic procedures.

EXPERIMENTAL

Sorbents

The silica used was macroporous glass MPS-2000-VGKh (Gorky, U.S.S.R.), with a particle diameter of 100–200 nm, a pore diameter of 220 nm and porosity of 1.6–1.8 cm^3/g. This macroporous glass was modified with DEAE-dextran in accordance with a previously described procedure for performing ion-exchange chromatography. For performing affinity chromatography, the glass was treated by silanization of silica with 3-glycidyloxypropyltrimethoxysilane (Fluka) by a described method [6].

Our general procedure for the preparation of a 10-g MPS-2000 tetanus toxoid column (15 × 1.6 cm I.D.) was as follows: 500 ml of tetanus toxoid (protein concentration 1.5 mg/ml) in 1 *M* KH_2PO_4 buffer (pH 7.0) was applied to the modified glass column and recycled for 24 h at a flow-rate of 50 ml/h. The column was then washed to remove uncombined ligand with 0.5 *M* KH_2PO_4 buffer (pH 7.0) and unreacted epoxy groups were blocked by treatment with 0.5 *M* glycine–NaOH buffer (pH 7.2) for 72 h. Finally, the column with the ready immunosorbent was washed with eluent (0.25 *M* acetic acid) and then with 0.2 *M* glycine–NaOH buffer (pH 7.2), containing 0.2 *M* NaCl.

Ultrogel AcA-34 was supplied by IBF (France).

Protein solutions

Tetanus toxoids, series 716 [specific activity 216 UL/mg (UL = Unit Linkage)], 664 (specific activity 274 UL/mg) and 909 (specific activity 243 UL/mg), were released from Mechnikov TsNIIVS.

A 4% solution of commercial normal human immunoglobulin prepared in this institute from donor's blood was used for further purification.

Chromatography

Liquid chromatography. This was carried out with a standard liquid chromatographic system (LKB, Bromma, Sweden) consisting of a Multiperpex pump (Model 2115), a Uvicord SII detector (Model 2838), a Superrac fraction collector (Model 2211), a glass column (100 × 2.6 mm I.D.) (Pharmacia, Uppsala, Sweden) and glass columns of our own construction with I.D. 1.6 cm and lengths from 15 to 25 cm, depending on the scale. For detection, the absorbance was measured at 280 nm with the LKB detector. In some instances the protein concentration in samples and eluate fractions was determined by Lowry's method.

Ion-exchange chromatography. The tetanus toxoid was purified on a column containing 15 g of DEAE-dextran–MPS-2000 conjugate, taking into account the negative charge of the molecule at pH 6.8–7.2 (p*I* 3.5). A 25 × 1.6 cm I.D. column was filled with an aqueous suspension of chemically modified MPS-2000 and equilibrated with 0.01 *M* sodium phosphate buffer containing 0.15 *M* NaCl (pH 6.8). An 80-ml volume of tetanus toxoid was passed through the column at a flow-rate of 150 ml/h. Toxoid adsorbed on the carrier was eluted with 0.01 *M* sodium phosphate buffer containing 0.4 *M* NaCl (pH 6.8). Fractions of 5 ml were collected and assayed for toxoid and protein contents.

Affinity chromatography. A 500-ml volume of 4% immunoglobulin solution in starting buffer was passed through the column at a flow-rate of 300 ml/h. After washing off uncombined protein, the tetanus antibodies were eluted with 0.25 *M* acetic acid at a flow-rate of 60 ml/h followed by diafiltration at pH 4.0 to a 1% solution in terms of protein (Fig. 1). Maltose was added to the ready preparation at a final concentration of 10% as a stabilizer.

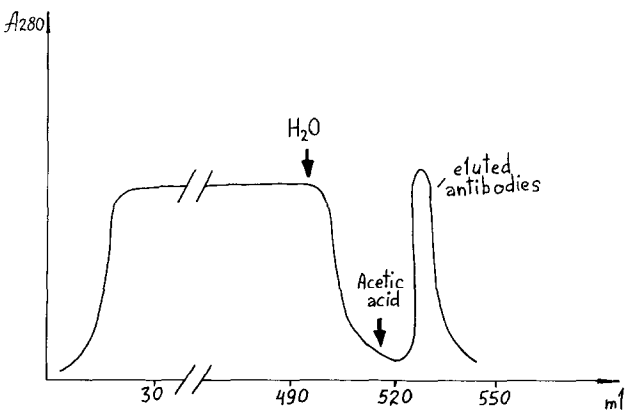

Fig. 1. Elution profile obtained for preparative affinity isolation of tetanus antibodies.

During storage the columns with modified glasses were always equilibrated with starting buffers containing 0.1% sodium azide after sterilization for 24 h in 2% formaldehyde.

Gel-permeation chromatography. This was carried out in a 100 × 2.6 cm I.D. column filled with Ultrogel AcA-34 equilibrated with 0.05 *M*. Tris–HCl buffer containing 0.15 *M* NaCl (pH 7.8). The buffer flow-rate was maintained at 30 ml/h. Elution profiles were monitored at 280 nm.

Analytical methods

Immunoelectrophoresis was performed by the rocket method according to Laurell [7]. Tetanus antibody levels were determined by an enzyme-linked immunoadsorbent assay (ELISA) technique [8] using as a standard human antitetanus immunoglobulin containing 125 IU/ml of specific antibodies, prepared by the NIIVS (Tomsk, U.S.S.R.) series 23.

Anticomplementary activity was measured by a method based on that described in ref. 9. Two-fold dilutions of the tetanus antibody solution were incubated with 2CH50 units of complement. Following overnight incubation at 4°C, 1% sensitized sheep blood cells were added and incubated at 37°C for 30 min. The anticomplementary activity was expressed as the amount of protein in milligrams in the dilution showing at least 50% haemolysis.

RESULTS AND DISCUSSION

Previous studies have shown that the content of tetanus antibodies in fourteen series of human immunoglobulins studied is 7.0 IU/ml [10]. It is obvious that the plasma of routine donors cannot be employed to prepare antitetanus immunoglobulins by Con's method but these preparations are suitable as a starting material for isolating immunoglobulins by affinity chromatography.

The initial studies were carried out on a DEAE-dextran–MPS-2000 column. Tetanus toxoid used as a ligand for obtaining antitetanus immunoglobulins contains impurities which should first be removed. In the U.S.S.R., the production of the commercial preparation uses a two-stage acid–salt method of toxoid purification. However, this is certainly inadequate for such processes as immunoaffinity chromatography, because constituents of the growth medium and metabolites tend to induce undesirable reactions. Different series of toxoid were each fractionated several times and the results from each run were essentially reproducible. An 80-ml sample of tetanus toxoid was passed through the column and the toxoid eluted from the column was obviously free from most of the contaminating impurities. Protein determinations indicated that only 55% of the protein contained in the original sample was recovered in the eluted fraction. Typical results are summarized in Table I.

Thus, owing to the removal of the minor component of toxoid, which was in the breakthrough volume in ion-exchange chromatography, with a 10% loss in activity it is possible to purify toxoid at least 2-fold, up to a specific activity of 436 UL/mg (Fig. 2).

Studies were undertaken in order to demonstrate that tetanus antibody could be purified on an MPS-2000 tetanus toxoid column. Typical results for tetanus antibody purification are presented in Table II.

TABLE I

PURIFICATION OF TETANUS TOXOID BY ION-EXCHANGE CHROMATOGRAPHY

Before purification			After purification				Purification factor	Yield in terms of activity (%)
Volume (ml)	Series	Specific activity (UL/mg)	Volume (ml)	Activity (UL/ml)	Protein (mg/ml)	Specific activity (UL/mg)		
80 ml toxoid	909	243	55	560	1.20	466.66	1.92	88
			58	543	1.18	460.15	1.89	90
			55	586	1.30	450.75	1.85	92
50 ml toxoid	664	274	80	295	0.55	536.36	1.96	93
80 ml toxoid	716	216	50	654	1.60	408.75	1.90	95
			50	632	1.55	407.75	1.88	91
			53	640	1.60	400.00	1.85	98
			55	580	1.40	414.30	1.92	92
			52	585	1.45	403.45	1.87	88
			60	540	1.30	415.40	1.92	94
					Mean:	436.35	1.90	92

Non-bound globulins represented more than 99% of the proteins without affinity for the column. Their antitetanous activity was regularly decreased 10–20-fold after one passage. The purification factor, *i.e.*, the ratio between specific activity after and before purification, varied from 720 to more than 1400 for a single cycle. The yield in terms of activity was based on recovered international units and amounted to

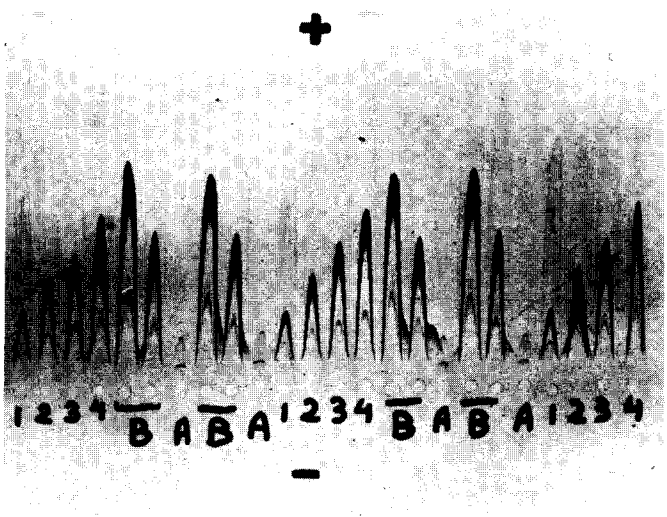

Fig. 2. Immunoelectrophoresis of the tetanus toxoids obtained by adsorption on DEAE-dcxtran–MPS-2000. Antigen: 5 μl filled the wells. Wells: 1–4, dilution of the standard tetanus toxoid; A, non-adsorbed toxoid; B, dilutions of the toxoid eluted from column. Antibodies: 0.5 ml equine hyperimmune, pepsin-treated, antitetanus γ-globulin diluted to 100 IU/ml + 10 ml of 1% agarose.

TABLE II

ISOLATION OF TETANUS HUMAN ANTIBODIES

Before purification		After purification				Purification factor	Yield in terms of activity (%)
Volume (ml)	Specific activity (IU/mg)	Volume (ml)	Activity (IU/ml)	Protein (mg/ml)	Specific activity (IU/mg)		
200	0.055	20	23.70	0.30	79	1430	90
425	0.055	15	75.00	1.14	66	1200	91
800	0.055	30	62.70	0.89	70	1270	90
500	0.068	16	34.50	0.70	49	720	85
300	0.063	20	26.00	0.40	65	1040	87
400	0.053	30	30.00	0.50	60	1132	90
425	0.053	20	41.45	0.63	66	1245	92
				mean:	65 ± 8.46	1148 ± 208	89 ± 2.5

90%. The activity yield was calculated by the ELISA test, which correlated well with the *in vivo* titre. The tetanus antibody solution prepared after the immunoaffinity column had a specific activity which was remarkably constant, being about 50–75 IU per mg of protein. Following 30 successive cycles, no reduction in the capacity or in the specificity of the column was detected.

A 1% solution has an activity of about 600 IU/ml, which remains constant for over 12 months without the formation of visible precipitates.

The anticomplementary activity of the preparations obtained, being at least 11 mg of protein per 2CH50, does not rule out the possibility of intravenous administration of tetanus antibodies. According to gel chromatography on Ultrogel AcA-34 (Fig. 3), the tetanus antibodies obtained are represented mainly by immunoglobulin G monomer (>90%).

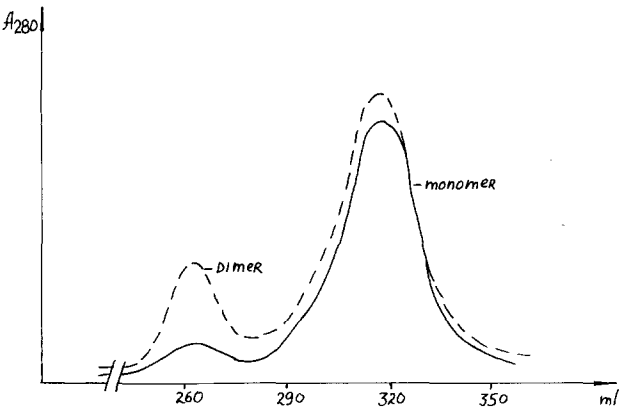

Fig. 3. Comparative gel-filtration analysis of immunoglobulin preparation on a column of Ultrogel AcA-34 (V_t = 430 ml, V_0 = 180 ml). Dashed line, normal donor immunoglobulin; solid line, tetanus antibodies.

In conclusion, using the isolation of tetanus antibodies as an example, we have experimentally substantiated the application of affinity chromatography based on modified macroporous silica for producing therapeutic immunoglobulin preparations (pure antibodies) from starting materials with very low initial concentrations.

The proposed method for the isolation of tetanus antibodies can be regarded as the most promising way of producing drugs with a selective action owing both to their exceptionally high specific activity and to the low anticomplementary nature of the preparations produced. The preparations produced in this way and containing pure immunoglobulins of the immunoglobulin G class will make it possible in the near future to discontinue almost completely the use of heterologous preparations in the prevention and treatment of tetanus.

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