Journal of Chromatography, 539 (1991) 355–362
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 905

Immobilized Cibacron Blue —leachables, support stability and toxicity on cultured cells

I. HULAK
IBF-Biotechnics, 35 Avenue Jean Jaurès, 92390 Villeneuve la Garenne (France)
C. NGUYEN
IMEDEX, B.P. 38, 69630 Chaponost (France)
and
P. GIROT and E. BOSCHETTI*
IBF-Biotechnics, 35 Avenue Jean Jaurès, 92390 Villeneuve la Garenne (France)

ABSTRACT

Although immobilized dyes are widely used on the laboratory scale and have good potential for industrial applications, they are still subject to some reservations. Little information is available about dye leakage and toxicity, which seriously hinders the use of such supports in the production of pure proteins. Investigations of the leakage mechanism and the *in vitro* toxicity of the native dye and of that leached from the column are reported. The possible presence of traces of dye in the purified biological materials necessitates the availability of sensitive analytical tests. The preparation and preliminary isolation of dye antibodies as a first step in the development of an immunohistochemical assay of leached dyes are also described.

INTRODUCTION

Chromatography is probably the most widely used method for the purification of enzymes and proteins. Interest is linked to the possibility of obtaining very pure substances, not only on the laboratory scale but also at the industrial level. Today, process chromatography is applied to the purification of many biological materials used in diagnostics and therapeutics.

It is obvious that at the level of industrial exploitation leading to the purification of a therapeutic product, the polymeric chromatographic support must be sufficiently stable and not release oligomers or other trace chemicals that could be toxic or have a secondary action. From this point of view, a number of experiments were performed in our laboratories to determine the level of leachables and undesirable products that might be present in the chromatographic supports.

An example of studies of leaching and toxicity is provided by immobilized dyes. Cibacron Blue F3 GA (Ciba Geigy) immobilized on a solid matrix provides adsorbents with high efficiency and specificity such that these supports are very attractive for application at the preparative level [1,2]. However, the dye may be leached under certain physico-chemical conditions and it is then necessary not only to determine the

0021-9673/91/$03.50

amount of ligand in the column effluents but also to establish its possible toxicity. This paper reports results on measurements of leached dye on agarose-based sorbents, the *in vitro* toxicity of the leached dye and preliminary results on the preparation and purification of specific antibodies against the dye.

EXPERIMENTAL

Native Cibacron Blue F3 GA was obtained from Fluka (Buchs, Switzerland), immobilized dyes on agarose-cross-linked beads from suppliers such as Pharmacia–LKB (Uppsala, Sweden) and Amicon (Danvers, MA, U.S.A.), foetal bovine serum culture media and all other chemicals for eukaryotic cell culture from Gibco-BRL (Paisley, U.K.) and biological materials such as bovine serum albumin (BSA), human haemoglobin and antibodies from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical-reagent grade. Chromatographic sorbents such as DEAE-Trisacryl Plus, protein A–Spherodex and Blue-Trisacryl were supplied by IBF-Biotechnics (Villeneuve la Garenne, France).

Determination and collection of leached dye

Agarose-immobilized Cibacron Blue was washed repeatedly on a Büchner funnel with water and packed into 50-ml columns. The sorbent was then perfused with acidic, neutral and alkaline solutions for several hours at room temperature and the effluents were collected. The dye present in the effluents was determined by spectrophotometry at 608 nm using a calibration curve from native Cibacron Blue. The sensitivity of this determination was close to 1 mg/l of dye.

In vitro cell culture

MRC-5 human cells were cultured in classical conditions in DMEM medium containing 10% foetal bovine serum at 37°C. Following WHO recommendations, MRC-5 cells were taken at the 29th passage and cultured over 25 days up to the 35th passage. Parallel cultures were realized: in the absence of any additive (standard culture), in the presence of 25 μg/ml of native Cibacron Blue and in the presence of 25 μg/ml of leached dye from agarose beads. Growth curves were determined by counting the cells and by calculating the cell division rate. The morphology of MRC-5 cells was also checked during the *in vitro* culture.

Determination of polyploidia level

At the 36th passage and after 72 h, cultured MRC-5 cells were submitted to another passage during which the mitosis were blocked. Under these conditions and after a hypotonic shock, chromosomes were fixed and spread out, then submitted to an acidic hydrolysis and subsequently stained. The proportion of polyploidic cells was determined on each culture slide.

Determination of in vitro genotoxic effect

The genotoxic effect of free Cibacron Blue was determined using the SOS-Chromotest. The standard genotoxic agent used was 4-NQO (4-nitroquinoline oxide) as recommended by the *in vitro* test supplier.

Preparation of polyclonal antibodies

Polyclonal antibodies of Cibacron Blue were prepared using a protein–dye conjugate. The free dye is actually non-antigenic and the preparation of antibodies can be accomplished only with an appropriate carrier.

As carrier we used BSA, which was first mannosylated and then couped with the dye. The latter operation was effected by simple contact of albumin and the dye (Cibacron is a preactivated dye) under alkaline conditions overnight. The solution was then neutralized and desalted by gel filtration through a Trisacryl GF 05 column to remove the excess of free dye. The resulting albumin–dye covalent complex was used for the immunization of rabbits under classical conditions and in association with Freund's additive. The methodology of immunization used was classical: injection of 1 mg of antigen per week for 3 weeks. Another injection was also effected after 3 weeks of rest. The antibodies were detected in the rabbit blood by an enzyme-linked immunosorbent assay (ELISA) test using as competitor a complex of human haemoglobin and Cibacron Blue obtained as described above for BSA.

Purification of Cibacron Blue antibodies

The chromatographic strategy followed to separate antibodies from hyper-immunized rabbit serum was based on three principles: whole rabbit immunoglobulins G (IgG) could be separated from plasma in one step on DEAE-Trisacryl Plus as previously described [3]; whole rabbit immunoglobulins G could be separated specifically in a single step using immobilized protein A; and antibodies against Cibacron Blue should be biospecifically adsorbed on immobilized dye and then separated from other proteins.

In the first series of trials rabbit immunized serum was injected directly onto a column of DEAE-Trisacryl Plus previously equilibrated with 0.025 *M* Tris–HCl buffer (pH 8.5) containing 25 m*M* sodium chloride. Different fractions obtained under a salt elution gradient were collected and then analysed (Fig. 3A).

In a second series of trials, an affinity purification was effected on a column of protein A–Spherodex. The whole immunized rabbit serum was first diluted with an equal volume of 1.5 *M* glycine NaOH buffer containing 2 *M* sodium chloride (pH 8.9) and then injected into the affinity column previously equilibrated with the same buffer. After washing, the adsorbed immunoglobulin G was eluted by injecting 1 *M* acetic acid (Fig. 3B).

As a third approach, a column containing 7 ml of Blue-Trisacryl was equilibrated with phosphate-buffered saline (PBS) and then the whole immunized rabbit serum was injected after PBS equilibration (2 ml of rabbit serum). After washing with PBS until elimination of non-adsorbed proteins, a first elution was done using 0.1 *M* sodium chloride. A second elution using the same buffer containing 1 *M* sodium chloride followed (Fig. 3C). All the fractions collected from the three columns were analysed by gel electrophoresis and by Ouchterlony double diffusion [4] against Cibacron Blue-substituted haemogolobin and BSA.

RESULTS AND DISCUSSION

It has been clearly demonstrated that most of the leached dye resulted from the acidic washings of the agarose-based sorbents. The amount of leached dye was

between 0.2 and 1.7 μg per millilitre of sorbent (depending on the pH). In comparison, a dye coupled to a synthetic sorbent did not show any leakage effect when exposed to the same acidic conditions. This phenomenon was correlated with the sensitivity of agarose to acidic media (pH < 4) as demonstrated previously [5]. An NMR spectrum of the column effluent showed that the dye containd a certain number of osidic linkages (*i.e.* linkages between sugars) related to the presence of a glycosidic part on the dye [6] (results not shown). This result indicates that the dye leakage was in fact the result of partial hydrolysis of the agarose chain supporting a molecule of dye covalently immobilized.

The *in vitro* cell culture of MRC-5 human cells showed that the lethal dose of dye was *ca.* 200–250 μg/ml. At this concentration, the cells did not adhere to the surface of the dish and then died. This is why we used 25 μg/ml of dye in the culture medium, which was compatible with cell adherence. At this concentration, the MRC-5 culture study showed a significant decrease in the division time when the medium contained 25 μg/ml of leached dye (Fig. 1). The size of the cells at the end of the culture was smaller when compared with a standard culture (microscopic observation). Conversely, the native dye did not seem to influence the cell division. The growth curve was very similar to the standard one. In addition, no morphological modification was detected [6]. A chromosome analysis (polyploidia) of MRC-5 collected after the 36th passage (after 22 days of culture) over thousands of metaphases indicated that no modification was induced in the presence of the native Cibacron Blue. The polyploidia level was actually of 6/512 (average) and was very close to the standard cell culture (7/520 on average). Both results were significantly lower than the WHO reommendations (17/520). The polyploidia level of the cells cultured in the presence of leached dye was significantly higher, however, above the limits of acceptance (see Table I).

Genotoxic studies performed with the SOS-Chromotest indicated the absence of any toxicity at concentrations far higher than those usually utilized. The dye genotoxic activiy, when compared with that of 4-NQO, was about three orders of magnitude lower (Fig. 2).

These studies were carried out at high concentrations of dye (from 25 to 500

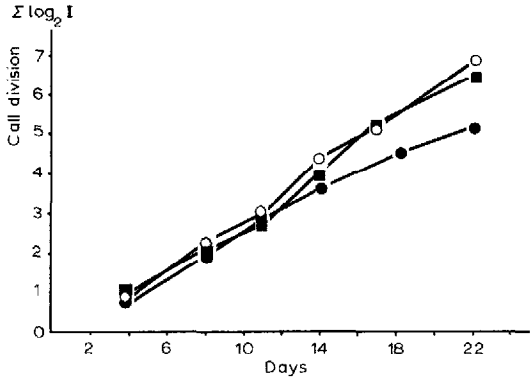

Fig. 1. Growth curves of MRC-5 human cells *in vitro* over six passages in classical conditions (from the 29th to the 35th passage). Cultures were effected in DMEM medium containing 10% of foetal bovine serum in the presence of 25 μg/ml of (■) native Cibacron Blue (NCB) or (●) leached dye (LCB). (○) Standard curve obtained in the absence of dye (FBS).

TABLE I

POLYPLOIDIA RESULTS FOR CULTURED MRC-5 CELLS IN THE PRESENCE OF CIBACRON BLUE AT THE 36th PASSAGE

Slide No.	Standard curve	MRC-5 + NCB[a] (25 μg/ml)	MRC-5 + LCB[b] (25 μg/ml)
1	9/538	5/536	23/528
2	5/503	6/503	15/512
3	8/510	9/513	17/522
4	5/529	5/502	18/508
Average	7/520	6/513	18/517

[a] NBC = Native Cibacron Blue.
[b] LBC = Leached Cibacron Blue.

μg/ml) whereas the amounts that possibly leaked during a chromatographic run were usually less than 1 μg/ml.

These preliminary data made it clear that the toxicity studies whould be extended to other determinations *in vitro* and/or *in vivo*. However, to improve the accuracy of these investigations, we thought that a more sensitive method of dye detection was essential, because the spectrophotometry is not sensitive enough and there is no easy means of localizing the dye once it has been incorporated into the cell. Hence the preparation of antibodies against Cibacron Blue was investigated. It was found that when the dye coupled with BSA was injected directly into rabbits, no antibody response was detected. However, in association with Freund's additive, a positive response was obtained in three out of four rabbits. The amount of secreted antibodies (determined empirically by measurement of the detection limit) was not constant from

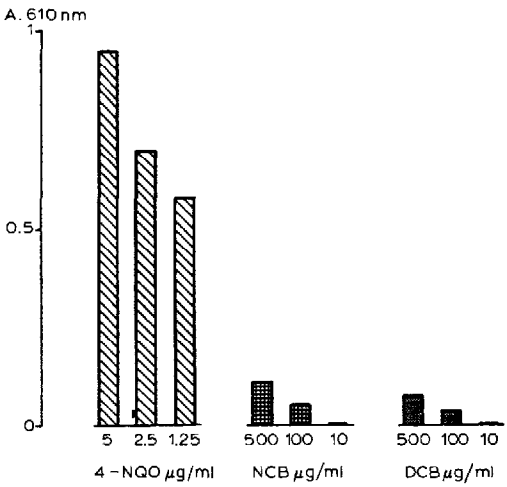

Fig. 2. Genotoxic results obtained using SOS-Chromotest. The standard genotoxic agent was 4-NQO. NBC = native Cibacron Blue; DCB = deactivated Cibacron Blue. The absorbance at 610 nm was the result of the final enzymatic activity induced by the genotoxic effect of the chemicals.

TABLE II

DETECTION DILUTION LIMITS OF SECRETED ANTI-CIBACRON BLUE ANTIBODIES

Days of immunization	Dilution limit of antibody detection	
	Rabbit 1	Rabbit 2
51	800	800
70	800	1600
85	800	3200
105	1600	3200
140	3200	6400
189	3200	N.D.

rabbit to rabbit (see Table II), but in more than 50% of the rabbits the secretion level was acceptable and increased week after week.

Table III and Fig. 3 show the main results of the isolation of anti-Cibacron Blue antibodies using three different approaches. Whole immunoglobulins G with good purity were obtained using DEAE-Trisacryl Plus, protein A–Spherodex or Blue-Trisacryl. In the first instance, most of the IgG, including specific Cibacron Blue antibodies, were present in the flow-through as expected [3]. This fraction contained only a few contaminants in small amounts; the purity determined by electrophoresis was in fact 95%. In the second instance the IgG fraction, obtained by acidic elution, contained most of the rabbit IgG and all Cibacron Blue antibodies. The purity of this fraction was similar to those obtained by ion exchange; however, the yield was higher (about 60%) than in the DEAE-Trisacryl Plus fractionation.

Blue-Trisacryl allowed us to obtain fairly pure antibodies (about 95% by electrophoresis) in one step using the described conditions. The fraction containing the Cibacron Blue antibodies was obtained by elution with 0.1 *M* sodium chloride. This fraction was not contaminated by the presence of albumin, which is known to have a high affinity for Cibacron Blue [7,8]; albumin was actually eluted quantitatively by 1 *M* sodium chloride washing. The agarose double diffusion experiments show that the

TABLE III

MAIN RESULTS ON THE SEPARATION OF IgG FROM IMMUNIZED RABBIT BY VARIOUS CHROMATOGRAPHIC PROCEDURES

Protein fraction	Chromatography	IgG purity (average) (%)	Presence of Cibacron Blue antibodies	IgG Yield (average) (%)
Whole serum	–	18–20	+	100
Flow-through DEAE-Trisacryl	Ion-exchange	95	+	>40
Fraction adsorbed on protein A-Spherodex	Affinity Fc fragment	95	+	>60
0.1 *M* NaCl elution on Blue-Trisacryl	Affinity Fab fragment	90	+	>90

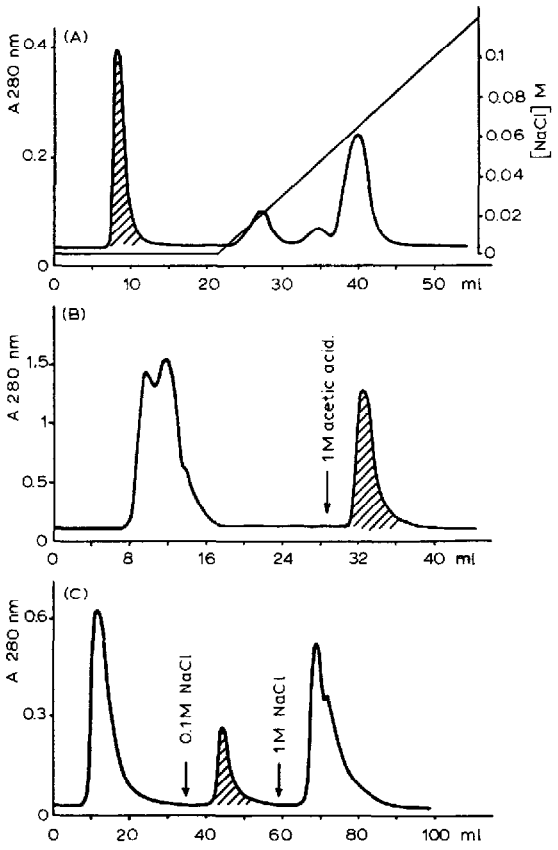

Fig. 3. Chromatographic profiles of IgG separation from immunized rabbit serum against Cibacron Blue. (A) Fractionation obtained by ion-exchange chromatography on DEAE-Trisacryl Plus; (B) fractionation obtained by affinity chromatography of IgG on protein A–Spherodex; (C) fractionation of anti-Cibacron Blue antibodies by affinity chromatography on Blue-Trisacryl. The sample in all instances was whole rabbit serum, previously equilibrated with the starting buffer. The grey areas indicate the localization of anti-Cibacron Blue antibodies. For further details, see Experimental.

anti-Cibacron Blue antibodies present in the flow-through of the DEAE-Trisacryl Plus column and in the absorbed fraction of protein A–Spherodex were contaminated by anti-BSA antibodies and other immunoglobulins. These sorbents in fact do not discriminate between the different categories of immunoglobulins G. On the other hand the Blue-Trisacryl active fraction (Fig. 3C) did not show any cross-reaction with BSA, demonstrating the absence of BSA antibodies. The latter were found in the flow-through of this column.

These antibodies of different purity will be used for the preparation of an ELISA-like or immunometric detection assay for free and leached dye from specific affinity sorbents.

CONCLUSION

This preliminary work demonstrated that at acidic pH, the agarose-based immobilized dyes are not stable. This instability seems to be related more to the matrix instability than to linkage breaking between the dye and the support. The leaked dye, which seemed to be associated with a small part of polysaccharide, modified the growth rate of the *in vitro* human MRC-5 cell culture and increased significantly the number of polyploidic cells. As these effects were not observed with a free native dye, a preliminary conclusion could be made that the osidic part of the leached dye was responsible for the difference. At this stage, models of dyes coupled with sugars should be synthesized and submitted to similar tests. On the other hand, we demonstrated that it was possible to synthesize and purify active antibodies against dyes, which represents a useful alternative to a spectrophotometric assay for the determination of traces of dyes.

ACKNOWLEDGEMENTS

This work was partially supported by a grant from the French Minister of Research and Technology as a part of EUREKA project EU-384.

REFERENCES

1 R. K. Scopes, *Anal. Biochem.*, 165 (1987) 235.
2 R. F. Sherwood, R. G. Melton, S. H. Alwan and P. Hugues, *Eur. J. Biochem.*, 148 (1985) 447.
3 G. Corthier, E. Bochetti and J. Charley-Poulain, *J. Immunol. Methods*, 66 (1984) 75.
4 O. Ouchterlony, *Prog. Allergy*, 5 (1958) 1.
5 E. Boschetti in C. Doinel (Editor), *Protein Purification Technologies*, G.R.B.P., Paris, Vol. 3, 1988, p. 23.
6 S. Oulieu, F. Viliamier, M. Allary, E. Boschetti and C. Nguyen, preliminary communication, *Proc. Symp. Protein–Dye Interactions, Compiègne,* 1988, unpublished results.
7 J. Travis and R. Pannell, *Clin. Chim. Acta*, 49 (1973) 49.
8 J. Saint-Blancard, E. Boschetti, P. Riberon, P. Claudel, P. Girot and M. Allary, *Sci. Tools*, 32 (1985) 37.