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Purification Process Monitoring in Monoclonal Antibody Preparation: Contamination with Viruses, DNA and Peptide Growth Factors

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Abstract. Administration *in vivo* of monoclonal antibodies to humans is challenged by considerations regarding their safety. Contamination with viruses, potentially oncogenic nucleic acids and biologically active components like growth factors and hormones forms a serious point of concern in this respect. We have investigated the potential risk of viral contamination by measuring the reduction of 12 different viruses (after spiking) in the standard downstream purification process of ascitic fluid. Depending on the type of virus added and the purification step employed, the reduction of infectious virus particles varies considerably. The overall reduction ranges from about 10^3, observed for a member of the family of Papovaviridae, to more than 10^{12} for members of the families of Herpesviridae and Orthomyxoviridae.

Using hybridization analysis with a mouse (genomic) DNA probe, we show that the amount of residual DNA in ascitic fluids may also vary considerably, ranging from 75 ng/ml to 1 μg/ml. In crude preparations produced in cell culture, much lower DNA concentrations are found (0·3 ng/ml). When standard downstream purification procedures are applied to ascitic fluid, a significant reduction of residual DNA levels is observed in the purified monoclonal antibody preparations and in intermediate fractions. The overall reduction factors vary from about 10^3 to 10^4, which is also confirmed by spiking experiments with either purified DNA or crude chromatin-like DNA.

Using in-vitro cellular assays, we further show that peptide growth factors like PDGF and TGFβ are present in considerable amounts in ascitic fluids. The observed biological activities, however, are completely eliminated during the purification steps applied.

Introduction

The development of the lymphocyte hybridoma technology, introduced by Köhler and Milstein in 1975,[1] represents one of the most successful applications of somatic cell hybridization. Fusion of B-lymphocytes with myeloma cells has allowed the generation of stable hybrid cell clones (hybridomas) which secrete monoclonal antibodies (MAbs) of predefined specificity. This not only opened new areas in immunology, but also proved to be invaluable in virtually the whole field of biomedical research.[2] Especially for in-vivo diagnosis and a variety of therapeutic purposes, like antitumor therapy, immunomodulation and (passive) immunization, a large number of monoclonal antibodies are now available or currently being tested.

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As with other innovations in modern biotechnology, the widespread use of monoclonal antibodies is challenged by new considerations regarding their safety.[3] In particular, the possible hazards associated with their in-vivo use in man forms a serious point of concern. Possible side effects and complications may occur which justify carefully performed (pre)clinical toxicological studies, as well as an appropriate risk assessment. The latter will be required also by regulatory authorities to obtain approval for their actual use in medical care.

Three major sources of potential problems are associated with the administration of monoclonal antibodies: (i) impurities associated with the preparation or manufacture, (ii) inherent properties of the immunoglobulin molecule and (iii) the host response to the protein. In this paper we have investigated the first source, impurities. Possible contaminants of

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monoclonal antibodies include viruses, nucleic acids, biologically active substances like growth factors and other immunogenic components. The most notable concerns thus far have concentrated on contamination with pathogenic viruses and DNA. Contamination with viruses has occurred in a number of biologicals administered to humans in the past. This includes ALV and HBV in yellow fever vaccine, SV 40 in poliomyelitis vaccine and more recently HIV in human blood products. It is important therefore to demonstrate that the purification regime is capable of removing or inactivating (different types of) viruses, and EEC recommendations have recently been developed to validate the removal of viral contamination in biological products.[4]

A long-term cancer risk may be associated with heterogeneous contaminating DNA, especially if it were to contain potentially oncogenic coding or regulatory sequences (like in hybridoma cells). The significance of this is supported by experiments in which the transforming properties of certain oncogenic DNAs have been demonstrated in animal experiments *in vivo* (reviewed by WHO, 1987).[5] Based on these results, moreover, recommendations regarding acceptable amounts of residual DNA (100 pg) per single dose were formulated.

In the present report we have investigated the reduction of infectivity of different viruses in the standard downstream purification process for ascitic fluid. For this purpose, viruses representing 12 different families, which in terms of susceptibility may be expected to cover the whole range of viruses, were added to the respective purification procedure.

In addition we determined the DNA content of different monoclonal antibody preparations (some intended for use in humans), as well as of the crude ascitic fluids and culture supernatants from which they were derived. Reduction of DNA content during the purification process is further demonstrated by different spiking experiments, making use of either [^{32}P] or [^{3}H]-labelled DNA.

Finally, we present the results of an examination for the presence of mitogenic activity (growth factors) and more specifically TGFβ in ascitic fluid and in purified samples thereof.

Materials and methods

Purification of monoclonal antibodies from ascitic fluid

Samples of ascitic fluid (code: 1-22D7E6MAI) used in the described control experiments were obtained from routine large scale ascitic fluid productions (National Institute of Public Health and Environmental Protection: Laboratory for Bacterial Vaccines). The ascitic fluid sample (start volume 11 ml) was heat inactivated by incubation for 30 min at 56°C (step I), followed by centrifugation at low speed (1000 ***g***) for 15 min at 4°C (step II). Subsequently, a high speed centrifugation step was performed, 100 000 ***g***, 60 min, 0°C (step III). Then, per ml of cleared supernatant, 1 g DE-52 cellulose (Whatman International Ltd, Maidstone, U.K.) in 6 ml 0·01 M phosphate buffer, pH 6·0 was added. After stirring for 1 h at 20°C the suspension was centrifugated at 4500 ***g*** for 15 min at 20°C, and the supernatant collected and adjusted to pH 7·4 with 1 M phosphate buffer pH 7·4 (step IV). Purified immunoglobulin preparations (class IgG) were finally obtained by two consecutive sodium sulphate precipitation steps with (final) concentrations of 20% and 18% sodium sulphate respectively (steps V and VI). The pellets were recovered by centrifugation at 4500 ***g*** for 30 min at 20°C and dissolved in Phosphate Buffered Saline (PBS), pH 7·2 to the original volume of the ascitic fluid sample.

Spike experiments with viruses

To determine the reduction of viruses in the downstream purification process of ascitic fluid, preparations of infectious viruses were added to the individual steps in the purification process (repeated spike). Five mixtures of different viruses were added to the purification process samples to reduce the number of samples which had to be dealt with. Members of the following virus families were included in these mixtures: 1. Poxviridae (fibroma virus), Parvoviridae (canine parvovirus) and Picornaviridae (poliovirus type 1); 2. Coronaviridae (transmissable gastroenteritis virus of pigs) and Herpesviridae (herpes simplex type 1); 3. Rhabdoviridae (vesicular stomatitis virus) and Papovaviridae (polyomavirus); 4. Orthomyxoviridae (influenza virus type A) and Reoviridae (reovirus type 2); 5. Adenoviridae (canine adenovirus type 2) and Togaviridae (sindbis virus). The member of the Paramyxoviridae (measles virus) was tested single. The combinations of viral strains were chosen in such a way that interference in the measurement of the infectivity titers of the individual virus strains was avoided. From each virus strain 1 ml of concentrated infectious particles (^{10}log TCID titer, see Table 1) was added to 11 ml of ascitic fluid or subsequent intermediate fractions thereof. Before and after each purification step a volume of 1 ml was taken for each individual infectivity titration test and immediately frozen at −70°C. Suitable purified ascitic fluid was

added before each step (e.g. before step II: ascitic fluid treated for 30 min at 56°C) to maintain the constant sample volume of 11 ml. The virus samples taken prior to purification step IV were diluted in 0·01 M phosphate buffer before storage to obtain the same dilution factor before and after this purification step. To avoid a high sodium sulphate concentration, which would cause a cytotoxic effect interfering with the measurement of infectivity titers, the virus samples recovered after step V and before and after step VI were dialysed against PBS. The virus strains, the appropiate test method used for the measurement of the infectivity of the different strains, the cell types used in the infectivity tests and the infectivity titers in the stock solutions are depicted in Table 1.

Infectivity titration for viruses

Three different read out systems were used: cpe, immuno fluorescent antibody assay (IFA) and HA, as indicated in Table 1. For the assays using cpe as read out the following procedure was chosen. To estimate reduction in infectivity titers, the samples were tested in logarithmic or semi-logarithmic dilutions. These were made in Hepes Eagle maintenance medium, supplemented with 5% FCS, 100 IU penicillin and 100 μg streptomycin per ml. Virus dilutions in 50 μl volumes were inoculated onto microtiter culture plates (Costar), subsequently $1{\cdot}5 \times 10^4$ cells in a volume of 100 μl were added per well. The plates were then sealed and incubated for 3–6 days at 37°C in a moist 5% CO_2 atmosphere. Cytopathic changes were scored to determine virus titers.[6]

Table 1. Specification of the virus strains and read out systems used for measurement of the reduction of spiked viruses

Virus strain and reference	Infectivity titer of stock suspension*	Cell culture	Read out system
Fibroma virus†	6·5	RK13‡	cpe
Poliovirus, Mahony type 1	7·5	Vero‡	cpe
Canine parvovirus	6·5	A 72	IFA
Transmissible gastroenteritis virus, strain Perdue	5·6	PK 15‡	cpe
Herpes simplex virus, type 1, 63-3390 RIVM isolate	7·5	Vero‡	cpe
Vesicular stomatitis virus, strain San Juan	7·8	Vero‡	cpe
Polyomavirus ATCC VR 252	6·3	MEC	cpe
Canine adenovirus, Enduracell d-A2 vaccine§	5·3	MDCK‡	cpe
Sindbis virus, strain AR 339	7·7	Vero‡	cpe
Influenza virus A Philippines 3/82, RIVM isolate	5·7	Embryonated chicken eggs	HA
Reovirus 2 ATTC VR 231	5·5	Vero‡	cpe
Measles virus, strain Edmonston B	5·0	Vero‡	cpe

* Expressed in ^{10}log TCID 50/ml, except for Influenza virus A (per 0·1 ml).
† Lyomyxovax, Rhône Mérieux, Lyon, France.
‡ Cell line obtained from ATTC.
§ Norden Labs, Lincoln, Nebraska, U.S.A.

Virus dilutions to perform the IFA were made as described above. The virus dilutions (50 μl) were simultaneously inoculated with an aliquot of 200 μl (2×10^5 cells/ml) freshly trypsinized canine kidney cells (A 72 cells)[7] in lab-tek chambers (Miles). The fifth day after inoculation the cells were fixed with −70°C ethanol. Subsequently an indirect immunofluorescence test was carried out using a known positive and negative anti-canine parvovirus serum coupled with biotin, and avidine-FITC as conjugate. Fluorescence was read microscopically and virus titers were estimated.[8]

An HA test was carried out to determine the infectivity titers of influenza virus. For this purpose serial 0·5 log dilutions were made in PBS and 0·1 ml of these dilutions were inoculated *in duplo* in the allantoic cavity of 10 days embryonated chicken eggs. Three days later the eggs were chilled and the allantoic fluid was harvested. An HA test was carried out using the allantoic fluid with 0·5% chicken erythrocytes at 4°C. The titers were expressed as the reciprocal of the dilutions still showing HA.

Preparation of labelled DNA for hybridization analysis and spike experiments

Mouse myeloma DNA (cell line P3/X63-Ag8.653) was purified according to standard methods,[9] and labelled by nick-translation as described by Rigby *et al.*[10] using [α-^{32}P]dCTP (Amersham; specific activity 111 Tbq/mmol). Purification of labelled DNA was carried out by gel filtration (Sephadex G-50, Pharmacia), yielding a DNA preparation containing about $1–2 \times 10^8$ cpm per μg DNA.

To obtain [^{3}H]-thymidine incorporated DNA, about 8×10^6 hybridoma cells (code: 84/7 cpv 6–22–16) growing *in vitro* in log-phase under standard conditions, were incubated for 16 h with 3·7 MBq [^{3}H]-thymidine (Amersham, specific activity 2·6–3·1 TBq/mmol). Cells were harvested, washed twice with PBS, resuspended in deionized water and lysed by freeze-thawing using dry ice. This crude chromatin-like DNA preparation, which was used in the spike experiments, contained about 10^6 cpm per μg DNA.

Determination of DNA content and removal of spiked DNA

The amounts of contaminating DNA in ascitic fluid and (unspecified) intermediate fractions (obtained from Laboratory for Bacterial Vaccines), as well as in supernatant-samples of hybridomas cultured *in vitro* (obtained from Dr de Rie, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) were determined by hybridization analysis of purified DNA fractions. Purification was according to standard methods for eukaryotic DNA.[9] This included lysis by addition of SDS (final concentration 1%) and proteinase K (Boehringer; 200 μg/ml), several phenol/chloroform extractions and a RNAse treatment. Subsequently the purified DNAs were dialysed against TE-buffer. Low amounts of purified DNA from in-process samples were further concentrated by lyophilization, and dissolved in 300 μl deionized water. After denaturation, the DNA samples were immobilized on a membrane filter (Genescreen Plus; NEN Research Products) as indicated by the manufacturer, using a dot-blot apparatus (Minifold SRC-96, Schleicher & Schuell). DNA from ascitic fluid samples and in-process samples was spotted in various amounts. For calibration purposes, purified mouse myeloma DNA (code P3/X63-Ag8.653) was spotted in duplicate, the amounts ranging from 1 pg to 1 ng per dot. Prehybridization was for 2–4 h at 65°C in a mixture of 6 × SSC, 0·1% SDS, 5 × Denhardt's solution, containing 100 μg/ml denatured herring sperm DNA (Sigma).

Hybridization analysis was carried out for 16 h after addition of $0·5 \times 10^6$ cpm/ml [^{32}P]-labelled probe (nick-translated mouse myeloma DNA) to fresh hybridization mixture. Consecutively, filters were washed at low stringency in 6 × SSC/0·1% SDS (65°C), 3 × SSC/0·1% SDS (20°C), and 1 × SSC/0·1% SDS (20 °C) for 30 min each. After autoradiography (XAR-5X-OMAT AR film, Kodak), the actual DNA content of the samples was determined by visual comparison of the observed signal with that of the calibration spots.

To validate the downstream purification process for nucleic acid reduction, [^{32}P]-labelled DNA ($1–2 \times 10^8$ cpm/μg) was added either to the ascitic fluid (single spike) or to individual steps in the purification process (repeated spike). In the case of the ascitic fluid, the single spike was with 25 ng labelled DNA per ml ascites, and with individual steps spiking was with 7·2 ng labelled DNA per ml of sample. DNA reduction was also checked by addition of [^{3}H]-labelled chromatin (10^6 cpm/μg: 400 ng/ml sample) to the individual steps. The amounts of labelled DNA before and after the respective purification step were measured by either Cerenkov counting (^{32}P) or liquid scintillation counting (^{3}H).

Determination of mitogenic activity

Growth factor activity of ascitic fluid and purified samples thereof was determined by the use of sensitive bioassays specific for either PDGF or TGFβ.[11] PDGF activity was determined by growth stimulation of quiescent Swiss 3T3 fibroblasts, clone C7C2, as measured by [^{3}H]-thymidine incorporation. Cells

were plated in 24 wells microtiter plates at a density of $5{\cdot}0 \times 10^4$ cells/1·8 cm^2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). After 24 h the medium was replaced by 1 ml of a 1:1 mixture of DMEM and Ham's F12 medium, now supplemented with 0·5% FCS. After 48 h in this medium, the test samples were added in 100 μl binding buffer, and incorporation of [^{3}H]-thymidine (18·5 KBq/well added) into cellular DNA was determined between 8 and 24 h after sample addition. Data are expressed relative to stimulation by 9% FCS. All characterized isomers of PDGF induce [^{3}H]-thymidine incorporation to a level of approximately 80% of that of serum in this assay, with a half-maximum effect of approximately 1 ng/ml.[11]

Activity of type β TGFs was assayed by their ability to inhibit specifically the proliferation of mink lung CCl-64 cells. Cells were plated in 24 wells microtiter plates at a density of $5{\cdot}0 \times 10^3$ cells/1·8 cm^2 in DMEM, 10% FCS. After 8 h of incubation, test samples were added in 100 μl binding buffer, and after an additional 48 h of incubation, 18·5 KBq [^{3}H]-thymidine was added per well. Incorporation of radioactivity into cellular DNA was determined 16 h later. This assay detects all characterized TGFβ isomers, with a half-maximum effect at 70 pg/ml.[11]

Results

Reduction of spiked viruses

The reduction of spiked infectious virus particles in the downstream purification process of ascitic fluid was determined for 12 different viruses as shown in Table 2. The reduction obtained with the standard heat inactivation step (30 min at 56°C) ranges from zero with polyomavirus to more than 10^6 with the influenza type A virus. Centrifugation at 1000 ***g*** has a slight effect on the reduction of all viral particles. The reduction of infectious particles obtained after the centrifugation step at 100 000 ***g*** ranges from zero for type 1 poliovirus to 10^3 for reovirus 2. With chromatography using DE-52 cellulose the reduction ranges from zero for poliovirus to 10^4 for canine adenovirus. Precipitation with sodium sulphate, finally, has only a slight effect on all viruses tested. The overall reduction of infectious particles (see last column Table 2) thus ranges from about 10^3 for polyomavirus to more than 10^{12} for the herpes simplex and influenza viruses.

Determination of DNA-content

The quantity of residual cellular mouse DNA in crude ascitic fluids and purified MAb preparations was assayed by dot-blot hybridization on a membrane filter using a [^{32}P]-labelled probe. To determine the DNA content and the sensitivity of the assay, purified murine DNA standards ranging from 1 pg to 1 ng were also spotted in duplicate, and hybridized in the same experiment. A typical autoradiogram with standards and sample dots is shown in Fig. 1. The results demonstrate that dots containing 4 pg DNA can still be visualized (panel A). Furthermore, the recovery of DNA during the DNA-purification procedure ranged from 50–90%, as detected in the hybridization assay by addition of exogenous mouse DNA (see also samples 2(a–c); panel B). The DNA contents of the different samples tested are summarized in Table 3. The amount of residual DNA in ascitic fluids varies from 75 ng to 1 μg/ml. Purified monoclonal antibody

Table 2. Virus infectivity reduction factors during downstream processing

Virus/purification step	I	II	III	IV	V	VI	Overall
Fibroma virus	4·5	0·3	2·4	1·6	0·3	+0·4*	8·7
Polio 1 virus	5·6	0·2	+0·1	0·1	1·3	+0·2	6·9
Canine parvo virus	0·3	0·6	0·2	2·8	+0·7	0·3	3·5
Transmissible gastroenteritis virus	2·8	0·3	1·4	2·4	+0·2	+0·6	6·1
Herpes simplex 1 virus	4·6	0·0	2·8	2·8	1·1	0·8	12·1
Vesicular stomatitis virus	3·7	+0·4	0·3	2·8	0·8	0·1	7·3
Polyoma virus	+0·1	+0·4	0·5	2·8	0·2	0·0	3·2
Canine adeno 1 virus	≥5·0	0·1	0·4	4·0	0·2	+0·1	≥9·6
Sindbis virus	2·4	0·0	0·2	1·4	0·1	+0·2	3·9
Influenza A virus	≥6·0	0·5	1·5	4·0	0·0	0·0	≥12·0
Reo virus 2	3·3	0·5	3·0	2·6	1·2	0·0	11·6
Measles virus	≥5·0	+0·2	1·8	2·8	0·9	+0·9	≥8·4

* '+' sign indicates an increase in virus titer.
Reduction factors are expressed as reductions in ^{10}log titers.

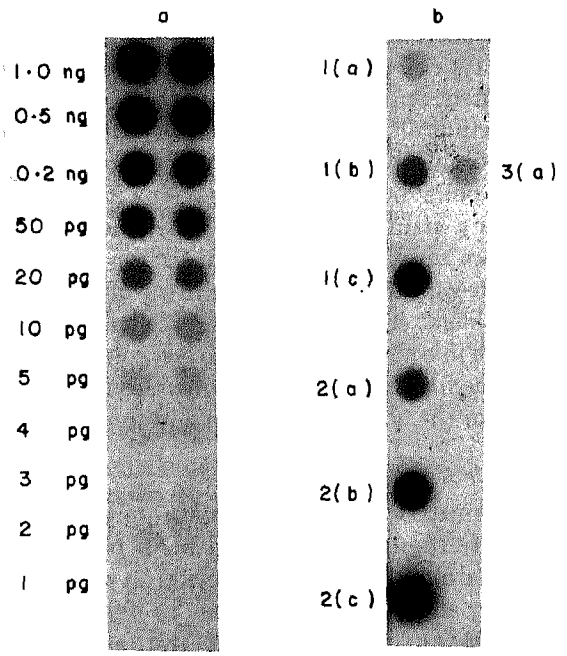

Figure 1. Autoradiogram of a dot-blot hybridization measuring residual DNA. Panel A: DNA standards spotted *in duplo*, containing 1 pg to 1 ng as indicated. Panel B: test samples with numbers referring to 1 (a–c), Sample 81/18 rabies, spotted in amounts corresponding to resp. 0·09, 0·45, 1·80 μl ascitic fluid; 2 (a–c), Intermediate fraction (CLBII) containing 1 μg exogenous mouse DNA/400 μl, spotted in amounts corresponding to resp. 0·08, 0·40, 1·60 μl sample; 3(a), Hybridoma cell culture supernatant corresponding to 133 μl sample.

preparations were found to contain 10–250 pg DNA per ml. The protein concentration of some of these purified MAb preparations has been determined by the supplying laboratory and correspond to 15–360 pg DNA per mg protein. Comparison of the DNA content of purified monoclonal antibody preparations and their corresponding ascitic fluids as shown in Table 3, reveals that a reduction of residual DNA by a factor of about 10^3–10^4 has occurred. Relatively low levels of contaminating DNA were measured in a supernatant of an in-vitro hybridoma cell culture. In this case about 300 pg DNA per ml was observed, and high speed centrifugation and filtration reduced the DNA content further to 60 pg/ml.[12]

Reduction of DNA-content

To validate the downstream purification process for nucleic acid removal, labelled DNA was added to ascitic fluid or to in-process samples. In the case of spiking with [^{32}P]-labelled DNA the amount of radioactivity before and after the separate purification steps was measured by Cerenkov counting, and the reduction factors were calculated after correction for differences in sample volume. The DNA reduction factors observed for the individual purification steps are shown in Table 4. Despite the differences in steps IV and V between the single and repeated [^{32}P]-spiking (first two columns), an overall reduction factor in the order of 10^4 is calculated in both cases. This reduction factor is in good agreement with the results obtained by the hybridization anal-

Table 3. Determination of DNA content by hybridization analysis and amount of residual DNA per mg purified MAb

	DNA content (ng/ml)			
Sample code	Ascitic fluid	Intermediate fraction	Purified MAb	pg DNA/mg purified MAb
WT 32 (1984)	1000	250	0·25	360
FK 18 (1982)	75	—	—	—
FK 18 (1985)	875	200	0·25	310
FK 6-12-20-1	—	—	0·10	110
WT32 (1986)	—	45	0·075	50
81/18 Rables	123	—	—	—
RIV9 lot 3	200	—	<0·02	<45
RIV9 lot 4	100–200	—	0·01–0·02	15–30
CLBII	—	1·25*	—	—
Hybridoma	0·3†	—	0·06*	—

— = Not determined.
* After high speed centrifugation and filtration (2 μm).
† No ascitic fluid but in-vitro culture supernatant.

Table 4. DNA and IgG3 reduction factors during downstream processing

Purification step	Reduction factor*			
	Single ^{32}P-spike	Repeated ^{32}P-spike	Repeated ^{3}H-spike	IgG3 RIV9†
II	1	1	1	—
III	9	11	3	3
IV	13	67	6	3
V	18	3	6	1
VI	8	6	15	2
Overall	16 848	13 266	1620	18

— = Not determined.
* Expressed in numerical values.
† Measured by means of ELISA.[15]

ysis above (Table 3). The DE-52 chromatography-step (IV) seems to be most effective in removing (repeatedly) spiked [^{32}P]-DNA. However, residual DNA in ascitic fluid will normally not be present as naked DNA but more likely complexed with proteins. Therefore we also examined the DNA removal after spiking with a more crude chromatin-like [^{3}H]-labelled DNA preparation. The results, as depicted in the third column of Table 4, show an overall reduction factor of 1620 which is about 10 times lower than the one observed with purified [^{32}P]-DNA. Furthermore, the last sodium sulphate precipitation step (VI) now seems to be the most effective step in removing the chromatin-like DNA. For comparison the reduction (loss) of IgG3, as typically observed in this kind of purification process, is shown in the last column of Table 4.

Determination of mitogenic activity and TGFβ

We have investigated whether the presence of mitogenic activity and more specific TGFβ, can be detected in ascitic fluid and in purified fractions thereof. Use has been made of two highly sensitive biological assays, one specific for PDGF-like growth factors, the major mitogen for connective tissue derived cells and related to the C-sis oncogene product, and the other for members of the TGFβ family.[11] Because of the assay requirements with regard to sterility, the purification steps were carried out in a somewhat different order. As shown in Fig. 2, strong

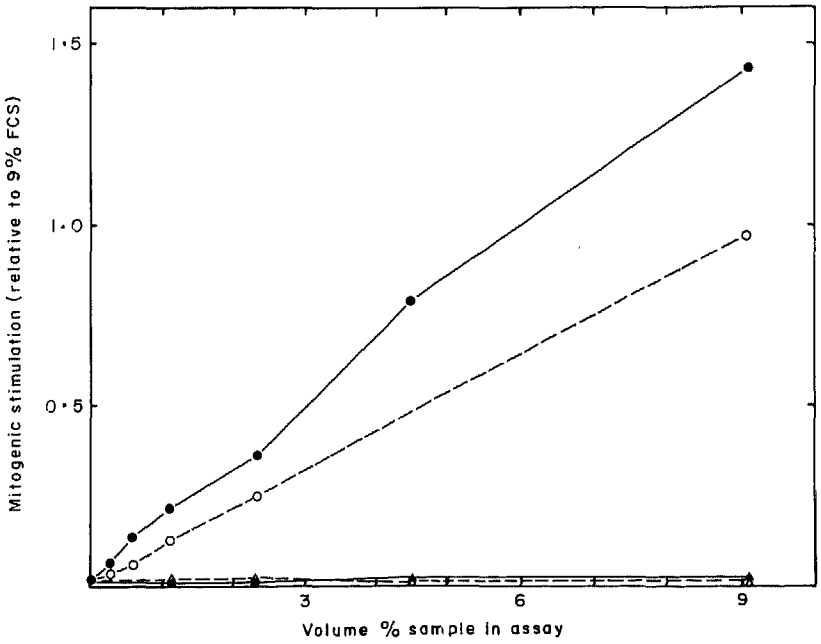

Figure 2. Mitogenic activity of ascitic fluid and purified fractions thereof. Determination of growth stimulating activity on quiescent Swiss 3T3 fibroblasts, and performance of purification steps were as described in Methods. Sample numbers refer to: 1. crude ascitic fluid after low speed centrifugation and without heat inactivation (●–●); 2. as 1, but dialysed against PBS (○–○); 3. as 1, with additional heat inactivation, high speed contrifugation, DE-52 chromatography (see Methods), and dialysed against PBS (▲–▲); 4. as 3, with additional sodium sulphate (2×) precipitation and dialysed against PBS (△–△). All data are expressed relative to stimulation by 9% FCS.

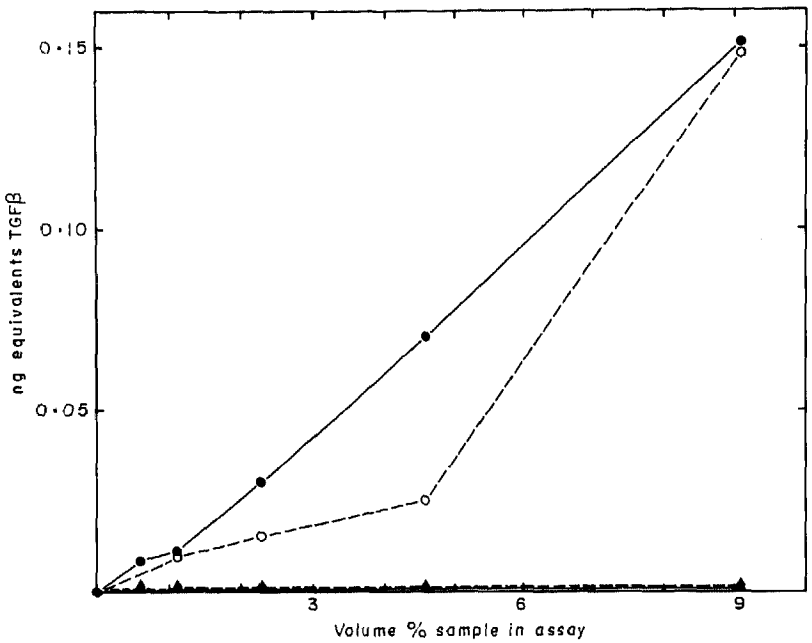

Figure 3. TGFβ content of ascitic fluid and purified fractions thereof. Determination of growth inhibition on CCl-64 mink lung cells was as described in Methods. Samples are as described in the legend to Figure 2. Data are expressed in ng equivalents of purified TGFβ.

mitogenic activity for Swiss 3T3 cells is present in samples 1 and 2, with stimulation levels at least as strong as foetal calf serum. This mitogenic activity, however, is completely removed during the purification steps applied to samples 3 and 4, indicating that no detectable PDGF activity remains present in those samples (detection limit assay 50 pg/ml).

Figure 3 shows that a comparable result is obtained when the same samples are assayed for TGFβ activity. Again, a considerable amount of active TGFβ is detectable in samples 1 and 2, while this activity is completely removed in samples 3 and 4 (detection limit assay 5 pg TGFβ/ml).

Discussion

The application of a relatively simple purification method to ascitic fluid is shown to be effective in reducing contaminating DNA, biologically active polypeptide growth factors and, although not all, infectious particles of spiked viruses.

The spiking experiments with virus particles reveal varying results depending on the virus strain used and the purification step applied. A broad range of model viruses, representative in terms of susceptibility for virtually all animal viruses, was included. The overall reduction of the different infectious virus particles observed in the standard purification procedure ranges from about 10^3–10^{12}. Since the contamination of ascitic fluid with certain viruses may well exceed levels of infectivity of 10^9, this may not be sufficient to completely remove all viruses. Viruses like the murine retroviruses were not included in our study, but other investigators have recently demonstrated at least a 10^{12}-fold reduction of spiked murine retrovirus during the purification of hybridoma cell culture supernatant.[18] Nevertheless, our results indicate that it is important to use animals for ascitic fluid production which are kept strictly isolated, derived from specified pathogen free colonies, and are regularly checked for viruses which may occur in mice.

Our results further show that hybridization analysis is capable of detecting residual DNA in crude ascitic fluids and purified MAb preparations with high sensitivity. This is in good agreement with the results reported recently by others for a similar method.[14] The amount of residual DNA in ascitic fluid was found to vary considerably, and this may be explained by differences in both cell density and percentage of lysed hybridoma cells. When the standard purification procedure is applied, a significant reduction of residual DNA levels is observed. Determination of DNA content in purified MAb preparations

as well as spiking experiments show that overall reduction factors vary from about 10^3–10^4. Compared with the 18-fold decrease in IgG3 content (Table 4) this indicates an efficient and specific purification process. The difference in overall DNA reduction factors may be explained by unequal amounts of protein complexed to the DNA. This is supported by the results obtained in the three different spiking experiments, especially when the individual purification steps are compared with regard to their effectivity.

Because necessary and essential information is still lacking,[5,16,17] quantitative estimations of the carcinogenic risk associated with the transfer *in vivo* of genomic (oncogenic) DNA can hardly be given at present. The results of an ongoing chronic carcinogenicity study in our institute in which the hybridoma DNA is administered subcutaneously to mice, will provide useful information in this respect. Nevertheless, given the DNA-contents measured and the reduction factors observed in this paper, the recent recommendations by regulatory authorities of ≤100 pg of DNA per dose, can easily be met. Furthermore, the risk of residual DNA is not only dependent on the amount, but also on the size of the DNA. In this respect it is important to note that relatively small DNA fragments were observed in ascitic fluid (≤0·5 kb, not shown).

An important feature of most cancer cells is autonomous growth, and this is reflected (among others) by their decreased dependency on exogenous growth factors. The obvious explanation for this is that cancer cells are capable of making and secreting (at least part of) the necessary growth factors themselves. This has been demonstrated for several tumor cell lines *in vitro*, giving rise to so-called autocrine loops for growth stimulation.[13] We have confirmed this for TGFβ by culturing the hybridoma cells 24–48 h in serum-free medium and examining the collected supernatant (not shown). The assay for mitogenic activity (Fig. 2) indicates the presence of a PDGF-like growth factor, most likely in synergism with other factors such as IGFs. Dialysis only partly reduces this mitogenic activity, as expected for these protein factors. However, virtually all mitogenic activity appears lost in the subsequent samples. Since the assay detects the combined and synergistic effects of different growth factors, it is not possible to determine a precise reduction factor in this case. Comparable results were obtained in the assay for TGFβ activity (Fig. 3). Also, here, the biological activity is no longer detectable in samples 3 and 4. This would imply a reduction factor of more than 30, assuming that all TGFβ is in its active form and given the detection limit of 5 pg TGFβ/ml. At present it is not well understood which of the treatment steps applied is responsible for the removal of the activity in both assays. Polypeptide growth factors are (relatively) heat stable and will not precipitate upon ultra-centrifugation. Also the DE-52 chromatography step, batchwise at pH 6·0 and intended to remove serum albumin, is not expected to bind growth factors like PDGF and TGFβ with isoelectric points above pH 10·0. Therefore, the removal may be due to non-specific adsorption, but this has to be investigated further.

The theoretical risks associated with oncogene-encoded proteins so far seem limited to growth factors and comparable biologically active proteins (cytokines). The effects of such peptide factors, however, are finite and reversible. Nevertheless, it cannot (yet) be excluded that repeated administration of large doses could generate a tumor-promoting effect.

Finally, the performance of an accurate and reliable risk assessment, as is urgently needed for this important new biotechnological innovation,[16–18] must be based on sound scientific judgement. Measurement of the reduced levels of important contaminants, as described in this paper, may provide part of the necessary data required for this.

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Appendix

AVL, avian leukosis virus; dCTP, deoxycytidine triphosphate; Denhardt, 1 × = 0·02% (w/v) Ficoll, 0·02% (w/v) Polyvinylpyrolidone, 0·02% (w/v) Bovine serum albumin; FCS, fetal calf serum; HBV, hepatitis B virus; HIV, human immunodeficiency virus; IGF, insulin-like growth factor; MAb, monoclonal antibody; MEC, primary mouse embyro cells; PDGF, platelet derived growth factor; SSC, 1 × = 0·15 M sodium chloride, 0·015 M sodum citrate; TE-buffer, Tris/EDTA (10mM/1mM) buffer; SV40, simian virus 40; TGFβ, transforming growth factor β.