The clinical pharmacology of biotechnology products
M M Reidenberg, editor

POSSIBLE PROBLEMS ASSOCIATED WITH CYTOKINE CONTAMINATION OF BIOTECHNOLOGY PRODUCTS

ANDREW GEARING*, MEENU WADHWA & ROBIN THORPE

*British Biotechnology, Watlington road, Cowley, Oxford, U.K. and NIBSC, Blanche Lane, South Mimms, Potters Bar, Herts, U.K.

INTRODUCTION

Animal cells have been used in the production of biological medicines for many years. Routine control testing of these products has emphasised elimination of contaminants such as host cell proteins in immunogenic quantities, host cell nucleic acids, micro-organisms, viruses or pyrogens (1). It has become clear in recent years that animal cells can produce a range of potent biologically active mediators known as cytokines (2). Cytokines are known to cause profound local and systemic inflammation, and to affect the function of most cell types. In view of this we undertook to investigate the production of cytokines by a number of cell lines commonly used to produce biological medicines, and also assayed their levels in several final products. This paper reviews our findings and assesses the potential risks and ways to minimise them.

MATERIALS AND METHODS

Cell lines. The cell lines were grown as indicated or as previously described (3). Supernatants were harvested from the lines by centrifugation either from confluent cultures of adherent lines or at the same time that product harvest would normally occur for suspension cultures. Ascitic fluid was also prepared from some of the hybridoma lines grown as ascitic tumours in mice.

Stimulation of cell lines. Lines were stimulated with IL1α 10IU/ml, LPS 10ng/ml or by addition of a number of wild type or vaccine strain viruses.

Cytokine assays. IL1 was measured using the NOB-1 bioassay which detects IL1α and IL1β with a sensitivity of 500fg/ml (4). IL6 was measured using the B9 bioassay which can detect 1pg/ml (5). TNFα and TNFβ were measured using the L929 bioassay which can detect 10pg/ml (6). GCSF and GMCSF were detected using two-site immunoassays (7)(Insight GM ELISA, MRL, Australia). Cytokine standards from NIBSC were used to calibrate the assays. 1 unit of IL1 corresponds to approximately 10pg, 1 unit of TNF to 25pg, 1 unit

of IL6 to 200pg, 1 unit of GCSF to 10pg and 1 unit of GMCSF to 100pg of recombinant proteins.

Vaccines and Recombinant Protein products. These were made up to their final dosage volume, or to 1mg protein/ml, prior to assay for cytokine levels.

RESULTS

Our initial experiments compared the levels of IL1, TNF, and IL6 in the culture supernatants of a variety of cell lines producing monoclonal antibodies (Table 1). Two were Epstein Barr virus-transformed human B lymphoblastoid lines, two were Heterohybridomas of EBV-transformed human B cells with a murine myeloma line, and ten were murine hybridoma cell lines. Both lymphoblastoid lines produced IL1 and TNF but not IL6 and one of the murine hybridomas produced IL6. None of the other lines produced detectable levels of any of the cytokines.

TABLE 1.
CYTOKINE LEVELS IN SUPERNATANTS FROM CELL LINES PRODUCING MONOCLONAL ANTIBODIES.

CELL LINE	IL1	TNF	IL6
EBV	7, 1	125, 16	0,0
HETEROHYBRIDOMA	0,0	0,0	0,0
HYBRIDOMA	0,0,0,0,0,0,0,0,0,	0,0,0,0,0,0,0,0,0,	0,0,0,0,0,0,0,0,0,2

All values are in U/ml, no GCSF or GMCSF could be detected in the EBV transformed lymphoblastoid lines or heterohybridomas.

We also compared the levels of cytokines in culture supernatants of the same murine hybridomas grow as ascitic fluid (Table 2). Nearly all of the ascitic fluid samples contained IL1, TNF and IL6. The highest IL6 levels were seen in the ascitic fluid of the line which made IL6 in culture.

A number of epithelial and fibroblast cell lines commonly used for the production of viral vaccines, or in the expression of recombinant proteins were cultured in IL1 or bacterial endotoxin (LPS) to determine their potential for cytokine release (Table 3). Most lines produced low levels of cytokines when unstimulated. The exceptions were CHO cells which made 3.7 U/ml IL6, Vero cells 11

U/ml of GCSF, and HELA cells 29 U/ml TNF. CHO cells constituitively release IL6, on some occasions up to 100 U/ml. Following stimulation with IL1 most lines increased production of IL6 and GCSF. MRC5 cells were particularly sensitive to IL1, secreting 100 U/ml of IL6 and 5760 U/ml of GCSF. LPS had negligible effects on most of the cell lines.

TABLE 2.
CYTOKINE LEVELS IN ASCITIC FLUID OF MURINE HYBRIDOMAS

HYBRIDOMA	IL1	TNF	IL6
1	1	10	2
2	3	9	1
3	1	3	1
4	1	1	2
5	1	0	1
6	0	1	2
7	0	4	0
8	1	1	0
9	1	6	15
10	2	6	1

All values in U/ml
Hybridomas used are same as in Table 1.

When MRC5, Vero and HEP2 cells were stimulated with a variety of virus strains a complex pattern of cytokine release was seen (Table 4) MRC5 cells could produce extremely high (ug/ml) levels of IL6 and GCSF in response to flu or mumps, and high levels of TNF in response to polio. Vero cells made high levels of IL1 and TNF in response to TypeII polio and High levels of IL6 in response to all polio types. HEP 2 cells made high levels of TNF in response to Type I and II polio and IL1 in response to Type II polio.

TABLE 3.
CYTOKINE PRODUCTION BY ENDOTHELIAL AND FIBROBLAST CELL LINES

CELL	STIMULUS	IL6	IL1	TNF	GCSF	GMCSF
MRC5	MEDIUM	0.1	ND	1.8	2.6	ND
	LPS	0.1	ND	0.4	2.6	ND
	IL1	100.2		0.3	5760.0	2.5
HEP2C	MEDIUM	0.2	0.1	ND	ND	ND
	LPS	0.4	0.1	ND	ND	ND
	IL1	0.3		ND	3.0	ND
HELA	MEDIUM	0.1	ND	29.0	ND	ND
	LPS	0.1	ND	3.2	1.6	ND
	IL1	0.3		44.0	2.1	ND
VERO	MEDIUM	1.2	ND	ND	11.0	ND
	LPS	6.3	0.1	0.3	26.0	ND
	IL1	6.0		ND	24.0	ND
CHO	MEDIUM	3.7	ND	ND	ND	
	LPS	4.3	ND	ND	ND	
	IL1	4.3		ND	50	

TABLE 4. STIMULATION OF CYTOKINE RELEASE BY VIRUSES

CYTO-KINE	CELL	FLU	POLIO I	POLIO II	POLIO III	MEAS	M V	M W	RUBELLA
IL6	VERO	45	2000	2750	3000	60	2	85	23
	HEP	23	40	30	12	1	1	1	1
	MRC5	35000	30	275	125	300	2000	10000	5
GCSF	VERO	80	ND	100	140	100	80	140	120
	HEP	1300	140	ND	ND	120	ND	100	280
	MRC5	10000	200	600	220	200	300	3500	350
IL1	VERO	3	12	120	37	6	1	9	ND
	HEP	2	1	70	1	6	2	5	2
	MRC5	4	ND	ND	ND	ND	ND	1	ND
TNF	VERO	ND	14	240	ND	5	6	3	6
	HEP	1	280	120	2	1	ND	ND	ND
	MRC5	3	144	144	29	ND	ND	ND	ND

LEVELS OF CYTOKINES ARE IN U/ML. ND = NOT DETECTABLE
Meas = measles, M V mumps vaccine strain, M W mumps wild

A number of viral vaccines were also found to contain cytokines, particularly IL6 and GCSF in high levels (Table 5).

The rabies vaccines were the worst preparations studied. None of the clinical grade recombinant proteins made in CHO cells contained significant levels, although one preparation of a laboratory reagent HIV gp120 had 8 U/ml of IL6.

TABLE 5.
CYTOKINE LEVELS IN VIRAL VACCINES AND RECOMBINANT PROTEINS

SAMPLE	IL6	IL1	GCSF
POLIO a	27	ND	ND
POLIO b	ND	ND	ND
RABIES a	800	7	30000
RABIES b	46	ND	ND
RABIES c	640	ND	2000
RABIES d	13	ND	ND
MEASLES	27	ND	ND
RUBELLA	25	ND	ND
MMR	4	ND	ND
CHO HEP	0.1	ND	ND
CHO EPO	ND	ND	ND
CHO TPA	ND	ND	ND

DISCUSSION

The initial impetus for this work came from the observation that an ascitic fluid of a hybridoma secreting antibodies which neutralised human IL1a contained something which stimulated the IL1 responsive cell line NOB-1(8). This turned out to be murine IL1. We then surveyed the production of IL1, TNF and IL6 in the culture supernatant of 10 different Hybridoma lines and in the corresponding ascitic fluid. The results demonstrate that hybridomas do not produce IL1 or TNF but one of the ten could make IL6. When grown as ascitic tumours the same hybridomas caused the secretion of IL1, TNF and IL6, this is presumably a host response to the tumour line. Some therapeutic monoclonal antibodies have been produced as ascites, our results would suggest that this method carries an increased risk of cytokine contamination, although some hybridomas may still produce IL6.

We extended our survey to include some human lymphoblastoid lines and heterohybridomas making human monoclonal antibodies. The lymphoblastoid cells produced IL1 and TNF but not IL6, whereas the heterohybridomas produced none of these. In a recent report we have also shown that lymphoblastoid lines can also make Transforming growth factor B1 and soluble CD23 (a B cell stimulating cytokine) (9).

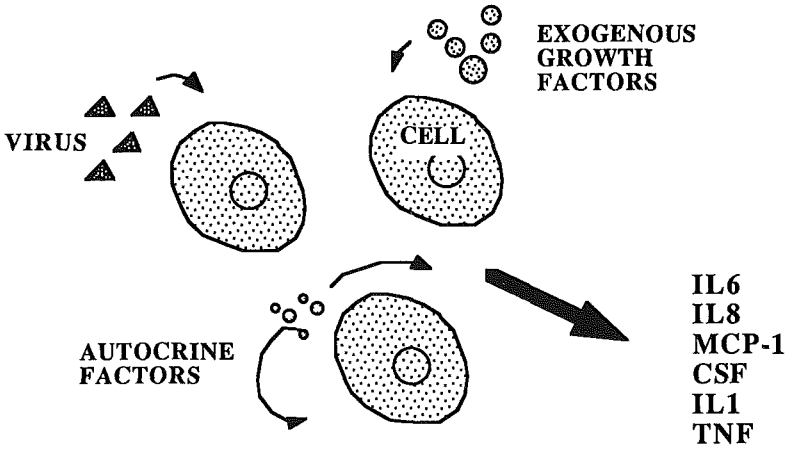
The epithelial and fibroblast lines showed differing patterns of constituitive and inducible cytokine release. Some such as MRC5 constituitively produced low levels of IL6, TNF and GCSF, others such as VERO produced high levels of GCSF, or HELA which made TNF and CHO which made IL6. Induction with IL1 markedly stimulated

MRC5 to make IL6 and GCSF, but had modest effects on the other cells. LPS had little effect. These results are in line with previous reports that LPS is not a potent stimulus for fibroblasts(10). Stimulation of cells with a range of viruses caused very high levels of cytokine release from some cell types. MRC5 cells produced 35000 units/ml(7 ug/ml)of IL6 in response to flu and 10000 units/ml (2ug/ml) in response to wild type mumps virus. Most cells also released IL1 and TNF. Van Damme and co-workers have also shown that viral stimulation of fibroblast lines causes similarly high secretion of both IL8 and MCP-1(11)(12). These are chemotactic and activating factors for granulocytes and monocytes respectively. Recent work also suggest that lines which are commonly used to express recombinant proteins for example COS can secrete soluble receptors for TNF, or CHO can secrete TGFB1.

Our work shows that many cell lines used in production of vaccines or recombinant proteins can secrete cytokines either constituitively, or in response to process components. These components can be the product itself as in the viral vaccines, or perhaps a biologically active protein which stimulates the cells directly. Other components include medium supplements such as growth factors which may also stimulate release. The extreme case would be growth as ascitic tumours in which the host animal produces cytokines as a protective response to the cell line. Cytokine content is not just a theoretical problem as we have shown that cytokines can be found in some clinically used vaccines (3).

The consequences of administering cytokines are variable, with local inflammation being the most likely problem. Some factors such as IL1 and TNF are pro-inflammatory, causing secondary release of many inflammatory cytokines. One hundred pg of IL1 injected intradermally into humans causes a marked inflammatory reaction (13). Other cytokines such as IL8 and MCP-1 are induced by IL1 or TNF and can themselves cause leucocyte infiltrations (14). Some factors such as GCSF and IL6 are not inflammatory. Systemic administration of IL1, TNF or high doses of IL6 can also be pyrogenic. The amounts of IL1, IL6 and TNF seen in most samples are however too low to cause fever in humans.

CYTOKINE PRODUCTION BY PROCESS CELLS



A more significant problem could arise if purification of a product caused co-purification of contaminating cytokines, doses could then reach toxic levels. A further consequence could also be the development of an antibody response against the injected cytokine. This would be more likely if the cell line used in production was non-human, and the cytokine was therefore of a different amino acid sequence. Cho cell can secrete high levels of IL6, and although this is not active on human cells, it could initiate an immune response to the hamster IL6 which could cross react with endogenous IL6. This would again only arise if some concentration of the cytokine had occurred.

Cytokines such as IL1 and IL6 may act as immunological adjuvants. This could be beneficial as in a viral vaccine but could be deleterious if antibodies to a therapeutic protein are formed. Avoidance of cytokine contamination is fortunately straightforward once it is known what cytokines are present in the process cultures. The physico-chemical properties of most cytokines are well known, and simple chromatographic procedures should remove most contaminants. An alternative approach would be to select cell lines which do not secrete cytokines as production cells. Sensitive biological, antibody and DNA probe assays are now widely available for most cytokines (15). A simple screen of process cells or samples should identify any particular cytokines and validate their removal on purification. If co-purification occurs then the purification system should be modified.

REFERENCES

1. World Health Organisation (1987) WHO Technical Report Series 747
2. Gearing AJH (1989) Thymus Update 2:127-145
3. Gearing AJH, Bird CR, Priest R, Cartwright JE, Wadhwa M, Thorpe R (1989) Lancet 1011-1012
4. Gearing AJH, Bird CR, Bristow A, Poole S, Thorpe R (1987) J. Imm Meths 99:7-11
5. Helle M, Boeije L, Aarden LA (1988) Eur J Immunol 18:1535-1540
6. Matthews N, Neale ML (1987) In: Clemens MJ, Morris AG, Gearing AJH (eds) Lymphokines and Interferons: A Practical Approach. IRL Press, Oxford, pp 221-225
7. Wadhwa M, Thorpe R, Bird CR, Gearing AJH (1990) J Immunol Meths 128:211-218
8. Gearing AJH, Leung H, Bird CR, Thorpe R (1989) Hybridoma 8:361-367
9. James K, Gardner J, Skibinski G, McCann M, Thorpe R, Gearing AJH, Gordon J (1990) Human Monoclonal Antibodies and Hybridomas In Press
10. Van Damme J, Schaafsma MR, Fibbe WE, Falkenburg JHF, Oppdenakker G, Billiau A (1989) Eur J Immunol 19:163-168
11. Van Damme J, Decock B, Conings R, Lenaerts J, Opdenakker G, Billiau A (1989) 19:1189-1194
12. Van Damme J, Decock B, Lenaerts J, Conings R, Bertini R, Mantovani A, Billiau A (1989) Eur J Immunol 19:2367-2373
13. Dowd PM, Camp RDR, Greaves MW (1988) Skin Pharmacol 1:30-37
14. Rampart M, Van Damme J, Zonnekeyn L, Herman AG (1989) Am J Pathol 135: 21-27
15. Gearing AJH, Cartwright JE, Wadhwa M (1990) In Press in Thompson AW (Ed) Immunology and Molecular Biology of Cytokines Academic Press, London

Discussion - POSSIBLE PROBLEMS ASSOCIATED WITH CYTOKINE CONTAMINATION OF BIOTECHNOLOGY PRODUCTS

R.G. Werner

What is the detection limit in your assays?

A.J.H. Gearing

The detection limit varies from about 500 fg/ml for the IL1 bioassay up to about 400 pg/ml for the least sensitive, the GCSF assay. And that is all biologically active and confirmed with neutralizing antibodies. So it is not a contaminant that is stimulating the assays, it is a specific material.

A. Ganser

If one injects a cytokine, such as G-CSF or IL3, into an animal or into a patient one has to expect the induction of a whole cascade of events including production of IL6. Actually I wouldn't like to have IL1 in my product but if it is IL6, which I know will induce anyway, I wouldn't think it will be too detrimental for the patient.

A.J.H. Gearing

The problem is that once you know that there is a contaminant there you should really do something about it, and regulatory authorities would probably take that view. If you are injecting a microgram of IL6, almost undetectable levels of IL1 and a bit of TNF, together with a large amount of another protein the problem of adjuvanting, becomes a possibility. IL1 or TNF can produce local inflammation, even when injected in small quantities, so my opinion is that route of administration is important. If you give it IV then nothing would happen. There is not enough there to cause a problem in general.

W.M. Wardell

What you presented has considerable implications for costs of products, not only commercial products (where I imagine the costs of what you proposed would be absorbable) but also at the investigational level. If one were forced to absolutely avoid any levels of cytokine contamination, what fraction would that add to the cost of the product being prepared just for an IND? It is these costs early in the development that add so much to the cost of the final product, through interest charges.

A.J.H. Gearing

That is an impossible question to answer, but you should take into account that most people only work with very few cell lines. The capacity of most cell lines to produce cytokines is reasonable well known. The ones that are produced in higher levels are IL6, GCSF and a few others. If the expression levels of product are reasonably high is it relatively simple to achieve purity. It is when one does ridiculous things like adding virus or using ascitic fluid or silly production schemes that problems arise.

L. Gauci

You have concentrated on the problem of contamination with cytokines when using certain types of cells. Clearly eucaryotic cells can produce many other biologically active substances, and I am thinking of hormones in particular. Do you have any feeling about whether it is really going to make the use of mammalian cells too complicated?

A.J.H. Gearing

No I don't think it is going to be a significant problem. As I said, the only thing I could see being a real problem would be minor amounts of local inflammation. I think the amounts you are going to see compared with the amount of product that comes out in the end, unless they copurify, are going to be very small.

J.A. Galloway

Shortly after we began marketing human insulin (rDNA) we received reports of three or four cases of an arthralgia-myalgia-arthritis syndrome which was associated with an increase in the erythrocyte sedimentation rate and a mild normocytic anemia. Cessation of treatment with human insulin rDNA resulted in complete resolution of the disorder. Except for those initial cases, we received no further reports related to the use of human insulin rDNA. However, we encountered a similar syndrome in the clinical trials with human proinsulin. Since none of the patients treated with human insulin or proinsulin were rechallenged, we were unable to establish a causal relationship between these agents and the condition described.