JCI The Journal of Clinical Investigation

Binding of cytokines to pharmaceutically prepared human immunoglobulin.

M Svenson, … , M B Hansen, K Bendtzen

J Clin Invest. 1993;92(5):2533-2539. https://doi.org/10.1172/JCI116862.

Research Article

Pharmaceutically prepared IgG, pooled from sera of over 2,000 normal individuals, contained both monomeric and dimeric IgG. Each type of IgG bound 125I-labeled interleukin (IL)-1 alpha, IL-1 beta, IL-6, and tumor necrosis factor (TNF)-alpha. Increased binding to IgG was observed if 125I-IL-1 beta was denatured by heating to 39 degrees C. However, the binding of both nondenatured and denatured 125I-IL-1 beta was not inhibited by unlabeled IL-1 beta. In contrast, binding of 125I-IL-1 alpha, 125I-IL-6, and 125I-TNF alpha was inhibited by the corresponding unlabeled cytokine. Papain-digestion of IgG abolished binding of 125I-TNF alpha but failed to influence the displaceable binding of 125I-IL-1 alpha and 125I-IL-6. 125I-TNF alpha was a mixture of trimeric and monomeric forms, the latter being the predominant form at lower concentrations. The apparent saturability of 125I-TNF alpha was explained by a higher nonspecific binding of monomeric than of trimeric 125I-TNF alpha to IgG. The amounts of cytokine antibodies in IgG preparations would contribute approximately 2 micrograms anti-IL-1 alpha IgG and 1 microgram anti-IL-6 IgG per kg body wt during high dose immune globulin therapy. In conclusion, pharmaceutical preparations of human IgG contain specific and neutralizing, high affinity antibodies against IL-1 alpha and IL-6, but not against TNF alpha or IL-1 beta. There are significant methodological pitfalls that hamper detection of IgG autoantibodies against cytokines.

Find the latest version:

https://jci.me/116862/pdf

Binding of Cytokines to Pharmaceutically Prepared Human Immunoglobulin

Morten Svenson, Morten Bagge Hansen, and Klaus Bendtzen
Laboratory of Medical Immunology, Rigshospitalet University Hospital, DK-2200 Copenhagen, Denmark

Abstract

Pharmaceutically prepared IgG, pooled from sera of over 2,000 normal individuals, contained both monomeric and dimeric IgG. Each type of IgG bound ^{125}I-labeled interleukin (IL)-1α, IL-1β, IL-6, and tumor necrosis factor (TNF)-α. Increased binding to IgG was observed if ^{125}I-IL-1β was denatured by heating to 39°C. However, the binding of both nondenatured and denatured ^{125}I-IL-1β was not inhibited by unlabeled IL-1β. In contrast, binding of ^{125}I-IL-1α, ^{125}I-IL-6, and ^{125}I-TNFα was inhibited by the corresponding unlabeled cytokine. Papain-digestion of IgG abolished binding of ^{125}I-TNFα but failed to influence the displaceable binding of ^{125}I-IL-1α and ^{125}I-IL-6. ^{125}I-TNFα was a mixture of trimeric and monomeric forms, the latter being the predominant form at lower concentrations. The apparent saturability of ^{125}I-TNFα was explained by a higher nonspecific binding of monomeric than of trimeric ^{125}I-TNFα to IgG. The amounts of cytokine antibodies in IgG preparations would contribute ~ 2 μg anti-IL-1α IgG and 1 μg anti-IL-6 IgG per kg body wt during high dose immune globulin therapy. In conclusion, pharmaceutical preparations of human IgG contain specific and neutralizing, high affinity antibodies against IL-1α and IL-6, but not against TNFα or IL-1β. There are significant methodological pitfalls that hamper detection of IgG autoantibodies against cytokines. (*J. Clin. Invest.* 1993. 92:2533–2539.) Key words: human immune globulin, IgG • autoantibodies to cytokines • interleukin-1α • interleukin-6.

Introduction

Several reports have described the presence of antibodies against immunoinflammatory polypeptides (cytokines) in diseased as well as in healthy individuals (for review see reference 1). Since intravenous immunoglobulin therapy has been reported to be beneficial in more than 35 diseases with immunopathologic features (2), and because circulating cytokines may be involved in the clinical outcome of some of these diseases (3, 4), it is conceivable that at least part of the observed clinical responses may relate to administration of naturally occurring antibodies to some of these cytokines.

Different assays have been used to measure these antibodies: Western blotting (5–10), competition in RIAs and ELISAs (11–14), binding in direct ELISAs (8–10, 15, 16), binding of radiolabeled cytokines (12–14, 17–23), and biological interference assays (7, 8, 10, 15, 17, 24–27). However, other serum factors than antibodies may compete in RIAs and ELISAs, and inhibition of biological activities may be caused by many factors apart from neutralizing antibodies to cytokines (28–31). Judged by analyses of monoclonal antibodies there may not be a simple correlation between biological blocking and antibody reactivity in direct ELISAs or in Western blotting experiments, and detection of antibody binding may be sensitive or insensitive to minor alterations in, or denaturation of, the ligand (32, 33). Furthermore, it has recently been shown that different immunization procedures of rabbits using murine IL-1β resulted in production of antibodies that selectively reacted with nondenatured or denatured cytokine (34). It may therefore be argued that detection of antibodies against cytokines in humans should involve binding analysis to nondenatured and denatured forms of individual cytokines. In case of autoantibodies, ligand binding must be evaluated with regard to both saturable and nonsaturable binding, and the binding must take place at the Fab parts of the immunoglobulins.

To investigate this, we have analyzed the binding of the ^{125}I-labeled recombinant human cytokines IL-1α, IL-1β, IL-6, and TNFα to human IgG pharmaceutically prepared from the blood of healthy donors.

Methods

Six batches of human IgG (Nordimmun®) from the sera of at least 2,000 healthy Danish adults per batch were kindly donated by Novo Nordisk (Bagsværd, Denmark). The IgG was pharmaceutically prepared by gentle precipitation with polyethylene glycol followed by ion exchange and contains no chemically or enzymatically modified immunoglobulins. Human recombinant interleukin 1 (IL-1) α and -β were generous gifts from Dainippon (Osaka, Japan) and Novo-Nordisk (Gentofte, Denmark). Recombinant human tumor necrosis factor α (TNFα) was a gift from Ernst Boehringer Institute (Vienna, Austria). Recombinant human interleukin 6 (IL-6) was donated by Amersham (Birkerød, Denmark). ^{125}I-IL-1α (2–3 $\times$ 10^5 cpm/ng), ^{125}I-IL-1β (1–3 $\times$ 10^5 cpm/ng), ^{125}I-IL-6 (0.5–1 $\times$ 10^5 cpm/ng) and ^{125}I-TNFα (1–3 $\times$ 10^4 cpm/ng) were kindly provided by Amersham. All radiolabeled cytokines bound specifically to cell receptors with preserved intrinsic activity: ^{125}I-IL-1α and ^{125}I-IL-1β were tested on EL4 cells (35), ^{125}I-IL-6 was tested on B9 cells (14), and ^{125}I-TNFα was tested for cytotoxicity using WEHI 169 cells (36). Furthermore, molecular size chromatography of ^{125}I-IL-1α and ^{125}I-IL-1β showed less than 10% aggregation after storage at 4°C, whereas 15–20% of ^{125}I-IL-6 appeared as aggregates. Taken directly from the stock solution, more than 75% of ^{125}I-TNFα was on a trimeric form. Four different preparations of the ^{125}I-labeled cytokines were tested with similar results.

Rabbit antibodies to the Fc parts of human IgG (code 424) were from Dako (Glostrup, Denmark). Magnetized polymer beads coated with donkey anti-rabbit antibody (Amerlex-M, code RPN 510) were purchased from Amersham. The detergents Triton X-100 and Tween 20 were from Sigma (St. Louis, MO) and Merck (Darmstadt, Germany), respectively. Skimmed milk (lipid content below 1%) was purchased from a local supermarket.

Specific rabbit antiserum to human TNFα was obtained by repeated multiple-site intracutaneous injections of 10 μg human recombinant TNFα into high-responder rabbits (6). The antigen was coupled

Address correspondence to Dr. Klaus Bendtzen, Laboratory of Medical Immunology, 7544 Rigshospitalet University Hospital, 20 Tagensvej, DK-2200 Copenhagen N, Denmark.

Received for publication 11 January 1993 and in revised form 16 June 1993.

J. Clin. Invest.
© The American Society for Clinical Investigation, Inc.
0021-9738/93/11/2533/07 $2.00
Volume 92, November 1993, 2533–2539

with glutar aldehyde to diphtheria toxoid, added to Freund's incomplete adjuvant, and absorbed on $Al(OH)_3$ before immunization. Specific rabbit antiserum to IL-1β was made similarly, except that human recombinant IL-1β was heated at 39°C for 18 h before being coupled to diphtheria toxoid. The resulting antiserum bound native and heat-treated ^{125}I-IL-1β without cross-reactivity between the two forms.

RIA of ^{125}I-labeled IL-1β and TNFα. The rabbit antisera were diluted in 20 mM phosphate buffer, 125 mM NaCl, 0.1% NaN_3, pH 7.4 (PBS), containing 2% BSA (Sigma). The antisera were incubated in PBS, containing 2% BSA, with the ^{125}I-labeled cytokines for 3 h at 4°C and bound and free ligands were then separated by use of the Amerlex-M separation system (Amersham).

Binding of ^{125}I-labeled cytokines to human serum IgG and skimmed milk. IgG (Nordimmun®), and unlabeled and ^{125}I-labeled cytokines were diluted in PBS with 2% BSA. Coincubation was performed at room temperature in 100 μl, containing 6,000 to 40,000 cpm of ^{125}I-labeled cytokine, with or without 200 times excess of corresponding unlabeled cytokine (0.1–0.24 μg). Fifty percent binding of IL-1α and IL-1β to IgG occurs within 5 min (37). For practical reasons, however, coincubation of IgG with IL-1α, IL-6, and IL-1β was carried out overnight (18 h). Heat denaturation of IL-1β was carried out at 39–40°C for 18 h. Coincubation with TNFα was done for 72 h (see below). Binding of IgG to cytokines was either analyzed by molecular size chromatography or by second antibody precipitation. Saturation binding analyses with ^{125}I-IL-1α and ^{125}I-IL-6 to individual batches of pooled human IgG were performed in the following way: PBS, 100 μl, containing 2% BSA, 0,1% Triton X-100, 60 μg IgG, 500–5,000 cpm (=2–20 pg) of ^{125}I-IL-1α and variable amounts of IL-1α were incubated for 18 h at 4°C. Bound and free tracer were separated at 4°C on columns containing 500 μl of Protein G Sepharose CL-4B (Pharmacia, Uppsala, Sweden) with PBS and 0.005% (wt/vol) thiomersal as running buffer. The bound material was eluted with 100 mM glycine-HCl, pH 2.4. Unspecific binding was assessed in the presence of 200 ng IL-1α per 100 μl. Analysis of ^{125}I-IL-6 was done similarly, except for the use of 0.3 mg of IgG and 1,000–5,000 cpm (10–50 pg) of ^{125}I-IL-6 with variable amounts of IL-6. Unspecific binding was assessed in the presence of 700 ng IL-6 per 100 μl. The results were expressed in pM and as μmol cytokine bound per mol of IgG, calculated from a mol wt of 17.5 kD for IL-1α, 20.3 kD for IL-6, and 150 kD for IgG.

Binding of cytokines to 4% skimmed milk was performed in parallel with the above experiments, using the same concentrations of ^{125}I-labeled cytokines. The binding was assessed by molecular size chromatography as described below.

Chromatographic separation of IgG-cytokine preparations. All procedures were carried out at 4°C. The elution profiles of ^{125}I-TNFα coincubated with or without IgG (Nordimmun®) were determined by the use of a Sephadex G-100 superfine column (0.9 × 32 cm; Pharmacia) at a flow rate of 3.4 ml/h with fraction collection every 12 min. Separation of bound and free ^{125}I-labeled IL-1α, IL-1β, and IL-6 was performed on columns containing 10 ml of Sephadex G-75 superfine (Pharmacia). The eluting buffer was RPMI 1640 with 25 mM Hepes (Gibco Biocult, Paisley, Scotland), containing 0.14% BSA and 0.1% NaN_3, pH 7.2. Separation of IgG (Nordimmun®) was performed on a Sephacryl S200 superfine column (1.6 × 90 cm; Pharmacia) at a flow rate of 12 ml/h. Repetitive samples of 22 mg IgG in 600 μl PBS were applied. Selected fractions were pooled and concentrated in dialysis bags placed on polyethylene glycol 20,000 (Merck). The protein content was quantified by absorbance at 280 nm, using a standard IgG preparation in PBS as reference.

Second antibody precipitation of IgG-cytokine complexes. The samples were placed on ice and a previously determined optimal concentration of rabbit anti-human Fcγ antibodies was added. After 10 min, the samples were diluted 16 times in ice cold PBS, containing 2% BSA, and centrifuged at 2,000 *g* for 15 min at 4°C. The pellet activity and the total ^{125}I-activity were determined with an error of 4% or less in a gamma counter (1272 CliniGamma, LKB, Wallac OY, Finland). There was no binding of labeled cytokine to anti-Fcγ antibodies when coincubated on ice for 60 min followed by column chromatography.

Papain treatment of IgG. Purified IgG (Nordimmun®), 25 mg/ml in PBS, supplemented with 10 mM L-cysteine and 2 mM EDTA, was incubated with 20 mg papain-agarose (Sigma) under gentle agitation at 37°C for 18 h. The digested IgG was then applied on a Sephadex G-75 superfine column (Pharmacia). Fractions from the void volume to samples containing molecules of molecular size 35 kD were pooled and concentrated in a dialysis bag placed on polyethylene glycol 20,000 (Merck). The protein content was quantified by absorbence at 280 nm.

Results

Binding of ^{125}I-labeled human cytokines to serum IgG. As shown by others (38), the pooled human IgG preparation contained monomeric and dimeric IgG (Fig. 1). Fractions containing dimeric IgG or monomeric IgG were pooled for further use. As shown in Table I, dimeric IgG (I) and monomeric IgG (II) bound the ^{125}I-labeled cytokines. The displaceable binding of ^{125}I-IL-1α and ^{125}I-IL-6 was similar to dimeric, monomeric, and unseparated IgG. A higher total and displaceable binding of ^{125}I-TNFα was obtained with dimeric IgG. As shown in Table I, ^{125}I-IL-1β also bound to human IgG. Heat denaturation of the cytokine increased its binding almost seven times, particularly to dimeric IgG. The binding of ^{125}I-IL-1β, however, could not be displaced by 2 μg/ml of unlabeled IL-1β.

Binding of cytokines to papain-treated IgG. When comparing binding of cytokines to IgG before and after treatment of the antibody preparation with papain, more than 60% of the displaceable binding of ^{125}I-labeled IL-1α and IL-6 was preserved (Table II). Papain-treated IgG retained less than 10% of the displaceable ^{125}I-TNFα binding. In contrast to ^{125}I-TNFα and heat-denatured ^{125}I-IL-1β, ^{125}I-IL-1α and ^{125}I-IL-6 bound exclusively to fragments which were not precipitated by rabbit anti-human Fcγ antibody (Table II).

Effect of detergents and milk proteins on the binding of cytokine to human IgG. The presence of skimmed milk together with the detergents Tween 20 and Triton X-100 reduced the binding to IgG of the ^{125}I-cytokines and, as shown in Table III, only the displaceable amounts of ^{125}I-IL-1α and ^{125}I-IL-6 were retained on the IgG. Furthermore, the displaceable amount of ^{125}I-TNFα was reduced 4–10 times by addition of milk proteins, depending upon the detergent in the assay buffer (Table III). Nondisplaceable binding of heat-denatured ^{125}I-IL-1β to IgG was reduced almost 5 times.

Without detergent, the binding to 4% skimmed milk alone was 12% (^{125}I-IL-1α), 7% (^{125}I-IL-6), 25% (^{125}I-TNFα), and 60% (denatured ^{125}I-IL-1β). High ligand concentrations reduced the binding of ^{125}I-TNFα only. Thus, skimmed milk did

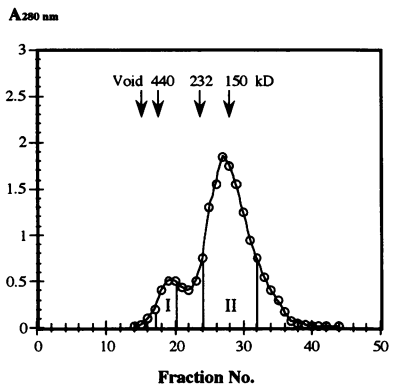

Figure 1. Sephacryl S200 molecular size elution profile of pooled human IgG (Nordimmun®). Fractions in each of the marked areas of dimeric (*I*) and monomeric (*II*) IgG were pooled and concentrated.

Table I. Binding of ^{125}I-labeled Human Cytokines to Sephacryl S200-fractionated, Pooled Human IgG

Cytokine	% binding				
	IL-1α	IL-1β		IL-6	TNFα
IgG:		A	B		
dimeric (I)	27 (25)	2 (−0.5)	14 (1)	20 (8)	12 (7)
monomeric (II)	29 (26)		7 (0)	16 (7)	8 (4)
unfractionated	28 (25)	1.5 (0.2)	9 (0)	17 (7)	7 (4)

Unfractionated dimeric and monomeric IgG, 3 mg/ml in PBS, were obtained by Sephacryl S200 chromatography (see Fig. 1). Binding was determined in triplicate as ^{125}I-activity precipitated by rabbit anti-human Fcγ antibodies. Results are shown as mean values of $cpm_{bound}/cpm_{total} \times 100$; numbers in parentheses are $cpm_{displaced\ with\ excess\ unlabeled\ cytokine}/cpm_{total} \times 100$. (*A*) non-heat treated traced and untraced IL-1β; (*B*) heat-denatured traced and untraced IL-1β.

not interfere with the binding of the cytokines to IgG by simple competition.

As shown in Table IV, the milk proteins also reduced binding of ^{125}I-TNFα and ^{125}I-IL-1β to their respective specific antibodies to a degree depending upon the structure of the ligand. However, this inhibition appeared to occur as a result of competitive binding to the milk molecules (Table IV).

Binding of ^{125}I-TNFα to pooled human IgG. Binding of ^{125}I-TNFα to IgG was carried out using 72 h of incubation before separation of bound and free ligand. This time of incubation was selected because molecular size separation of the tracer alone showed a time-dependent dissociation from an apparent trimeric to a monomeric form but with a stable distribution between the two forms after 72 h (data not shown). The distribution between the monomeric and trimeric structures of ^{125}I-TNFα was also concentration-dependent. In contrast, the relative content of higher polymers of ^{125}I-TNFα was not concentration-dependent (see Fig 2; void volume). Similar analyses using ^{125}I-IL-1α, ^{125}I-IL-6 as well as nondenatured and heat-denatured ^{125}I-IL-1β did not show a time- or concentration-dependent change in the distribution between the monomeric and polymeric forms of the cytokines.

Because of the concentration-dependent distribution of the monomeric and trimeric forms of ^{125}I-TNFα, binding to IgG was calculated with regard to both forms of the cytokine:

Table II. Binding of Cytokines to Papain-treated IgG

Cytokine	Preserved binding (%)			
	IL-1α	IL-1β*	IL-6	TNFα
Detection:				
chromatography with Sephadex G-100	76 (86)	17 (nd)‡	63 (62)	15 (8)
coprecipitation with rabbit anti-human Fcγ antibody	11 (2)	14 (nd)‡	17 (8)	12 (5)

IgG (Nordimmun®), 6 mg/ml in PBS, was pretreated with or without (control) papain. The effect is expressed as mean preserved binding of total bound ^{125}I ($n = 3$); the preserved displaceable binding is shown in parentheses. * Heat-denatured; ‡ nd: not detected.

Table III. Influence of Detergents and Skimmed Milk on the Binding of Cytokines to IgG

Cytokine		% binding			
	4% milk	IL-1α	IL-1β*	IL-6	TNFα
2% BSA	−	49 (41)	20 (−1)	29 (6.0)	12 (7)
	+	44 (40)	4 (0.2)	11 (6.7)	3 (1)
2% BSA	−		16 (1)		13 (8)
+1% Tween 20	+		3 (0.2)		5 (2)
2% BSA	−	55 (37)	26 (−1)	12 (6.7)	30 (22)
+0.1% Triton X-100	+	43 (38)	5 (0.5)	9 (6.3)	5 (2)

Human IgG (Nordimmun®), 6 mg/ml, was preincubated for 2 h in PBS with the additives indicated. The preparations were then incubated with labeled and with or without excess unlabeled cytokine. Binding of ^{125}I-labeled cytokines to IgG was detected by coprecipitation with rabbit anti-human Fcγ antibodies. Determinations were done in triplicate, and the results are shown as in Table I.
* Heat-denatured traced and untraced IL-1β.

$$cpm_{bound}/cpm_{free} = (a - b) \times cpm_{monomer}/cpm_{free} + b,$$

where $cpm_{bound} = a \times cpm_{monomer} + b \times cpm_{trimer}$

and $cpm_{free} = cpm_{monomer} + cpm_{trimer}$

As shown in Fig. 3, a highly significant linear correlation was obtained by plotting cpm_{bound}/cpm_{free} against $cpm_{monomer}/cpm_{free}$. This is obtained if a and b in the above equations are constants. Hence, it can be estimated from Fig. 3, that monomeric ^{125}I-TNFα exhibits more than 50 times higher nonspecific binding to IgG than trimeric ^{125}I-TNFα.

Individual batches of pooled human IgG bind IL-1α and IL-6 with high avidity. The equilibrium binding of IL-1α and IL-6 to a pool of human IgG as functions of the free ligand concentrations is shown in Fig. 4. IL-1α bound with an estimated average affinity (Kav) of 10 pM, whereas Kav for IL-6

Table IV. Skimmed Milk Suppresses Binding of ^{125}I-labeled TNFα and IL-1β to Specific Heterologous Antibodies

Skimmed milk	% suppression			
	IL-1β		TNFα	
	A	B	C	D
1%	4 (3)	30 (27)	7	35
4%	6 (4)	64 (50)	10	45

Specific rabbit antibodies against human TNFα and IL-1β were used. To obtain different relative amounts of monomeric ^{125}I-TNFα, the tracer was either taken directly from the stock solution (*C*) or preincubated for 72 h at 10,000 cpm/100 μl (*D*) (see Fig. 2). The tracer concentration during the binding assay was 4,000 cpm/100 μl. Without milk, 69% of the control and 55% of the preincubated ^{125}I-TNFα bound to the antibodies. The rabbit anti-IL-1β antibodies bound 43% and 50% at 5,000 cpm/100 μl of nontreated (*A*) and heat-treated (*B*) ^{125}I-IL-1β, respectively. The tracer solution was preincubated with milk for 2 h before addition of antibodies. Mean values of triplicate determinations. Values in parentheses: binding of ^{125}I-IL-1β before addition of antibodies. Results are derived from Sephadex G-75 chromatographic separations.

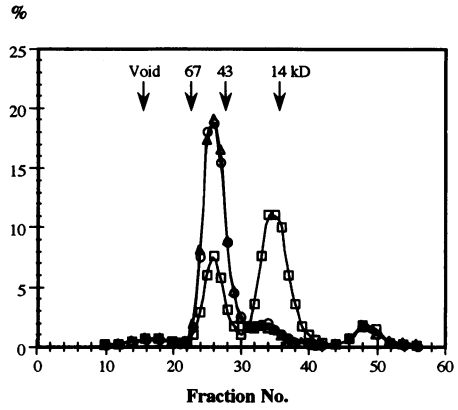

Figure 2. Elution profiles of ^{125}I-TNFα at different concentrations and after preincubation at room temperature. ^{125}I-TNFα, 3 μg/ml stock tracer solution, was diluted to 5 ng/ml in PBS, supplemented with 2% BSA; 150 μl was then applied on a Sephadex G-100 column at 4°C after 2 h (○) and after 72 h of preincubation (□). ^{125}I-TNFα, 5 ng/ml, preincubated with 2.5 μg/ml of TNFα for 72 h was also tested (△). The recoveries of tracer in the column runs exceeded 95%.

was 80 pM. In addition, the binding capacity (Bmax) of this IgG preparation was three times higher for IL-1α than for IL-6 (1.2 μmol/mol IgG vs. 0.38 μmol/mol IgG).

The different batches of pooled human IgG were almost identical with regard to IL-1α binding. In contrast, the Kav and, to a lesser extent, Bmax of the binding to IL-6 varied considerably between the IgG preparations (Table V). There was no cross-binding between IL-1α and IL-6 to the IgG preparations. Thus, 1 mg of the different batches of IgG contained 0.6–0.9 ng anti–IL-1α IgG and 0.2–0.4 ng anti–IL-6 IgG. Assuming that high-dose immune globulin therapy would contribute approximately 10 mg/ml to the preexisting level of IgG and noncooperative, single-site binding of the cytokines to IgG (2), the infused immunoglobulin would bind approximately 80% of IL-1α and 20–70% of IL-6 at a ligand concentration of 1 ng/ml (Fig. 5).

Discussion

A number of reports have appeared describing naturally occurring human antibodies against cytokines (5–8, 10–25, 27).

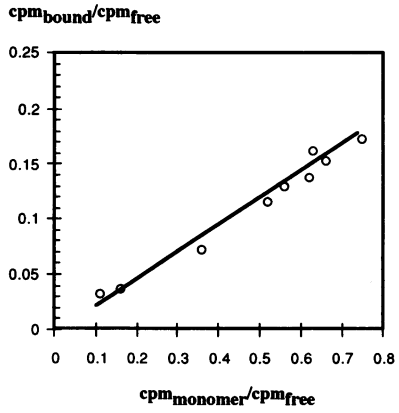

Figure 3. ^{125}I-TNFα binding to human IgG. IgG, 15 mg/ml PBS with 2% BSA, was incubated with 3 ng/ml to 2.5 μg/ml of ^{125}I-TNFα for 72 h at room temperature; 150 μl was applied on a Sephadex G-100 column at 4°C. The void volume cpm and the cpm distributed in the monomeric and trimeric pooled fractions of the ^{125}I-TNFα elution profile (see Fig. 2) were calculated. The recoveries of tracer in the column runs exceeded 95%. Cpm$_{bound}$ was calculated after subtraction of the void volume cpm of the tracer alone. The values were plotted as described in the text. Correlation coefficient = 0.98.

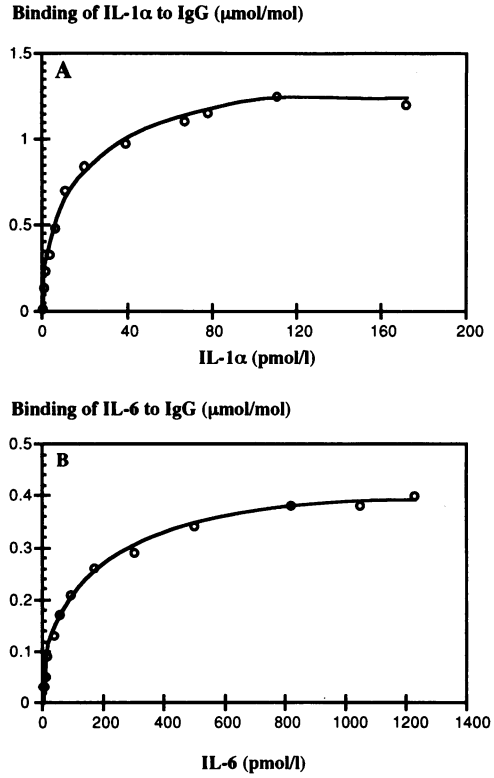

Figure 4. Equilibrium saturation binding of IL-1α and IL-6 to human IgG. Specific binding is shown as total minus nonspecific binding of duplicate determinations. Binding capacity (B_{max}) was estimated from the plateau of the curve and the average affinity (K_{av}) as the free ligand concentration at 50% of B_{max}. (*A*) IL-1α; K_{av} = 10 pmol/liter: B_{max} = 1.2 μmol/mol. (*B*) IL-6; K_{av} = 80 pmol/liter: B_{max} = 0.38 μmol/mol.

Some of these antibodies seem to occur relatively frequently even in apparently healthy individuals; see ref. (1). Thus, serum IgG directed against TNFα and IL-1α have been reported in 0–40% and in 1–30% of normal individuals, respectively, (5, 6, 9, 20–23). Recently, IgG specific for IL-6 were found in 15% of sera from Danish blood donors (14).

Saturation binding analysis is a necessary prerequisite if the presence of autoantibodies is being evaluated. In this regard,

Table V. IL-1α and IL-6 Binding to Individual Preparations of Pooled IgG

IgG batch no.	IL-1α		IL-6	
	K_{av}	B_{max}	K_{av}	B_{max}
	pmol/liter	*μmol/mol*	*pmol/liter*	*μmol/mol*
1	10	1.2	80	0.38
2	12	1.5	90	0.55
3	10	1.3	10	0.81
4	14	1.6	10	0.73
5	10	1.8	80	0.59
6	10	1.6	50	0.53

Six individual batches of Nordimmun® were tested for binding of IL-1α and IL-6 as described in Fig. 4.

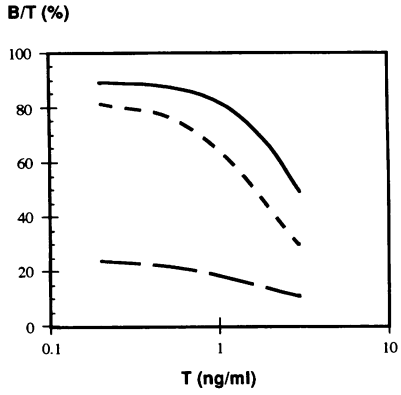

Figure 5. Estimated binding of IL-1α and IL-6 to 10 mg/ml of different batches of human IgG. Calculations were carried out using the formulas:

$$B/F = \frac{B_{max}}{K_{av} + F} \quad \text{and} \quad T = B + F$$

where B is the concentration of bound ligand;
F is the concentration of free ligand;
T is the total concentration of the ligand;
B_{max} is the total concentration of binding sites; and
K_{av} is the average affinity.

(——) IL-1α, batch 3, see Table V; (— —) IL-6, batch 1, see Table V; and (- - -) IL-6, batch 3, see Table V.

direct ELISA and Western blotting assays have limited use compared with binding analyses using ^{125}I-labeled cytokines in solution. This relates to the amount and state of denaturation of the fixed molecules and to possible differences in the binding properties of fixed and dissolved cytokine (32, 39).

In accordance with previous results using sera of normal individuals (13, 14, 19, 20), we found evidence of autoantibodies directed against IL-1α and IL-6 in all of six human IgG preparations manufactured for clinical use. Hence, the binding of these cytokines was saturable and selectively occurred to the Fab fragments of the IgG molecules. Also, the binding affinities of these antibodies were in the same range as those previously detected for IgG in individual human sera (20, 35).

Pools of human immune globulin have been shown to contain dimeric IgG, which has been proposed to constitute idiotype–anti-idiotype pairs (38, 40). However, compared with unfractionated and monomeric IgG, dimeric IgG did not differ in their specific binding of ^{125}I-IL-1α and ^{125}I-IL-6, indicating the absence of appreciable amounts of blocking anti-idiotype antibodies. This is supported by the observation that individual sera failed to block ^{125}I-IL-1α binding to a known amount of added human IL-1α antibodies; our unpublished findings, for reviews see reference 21.

Nonspecific binding may be reduced by the use of detergents (41), and milk proteins have been proposed to be superior to other proteins in blocking nonspecific binding of IgG in solid phase assay systems (42). Accordingly, skimmed milk reduced the nonspecific binding of both ^{125}I-IL-1α and ^{125}I-IL-6; the detergent Triton X-100 reduced only nonspecific binding of the latter. ^{125}I-IL-β exhibited low and nonsaturable binding to IgG. Heat denaturation of ^{125}I-IL-1β increased only the nonspecific binding, and milk proteins suppressed the binding of the cytokine. From the analyses of individual sera, we and others have not been able to obtain evidence of antibodies of the IgG class directed against nondenatured IL-1β (19–21). Our data further indicate that IgG specific for denatured IL-1β occur rarely, if at all, in sera of healthy humans.

The existence of natural IgG directed against TNFα in humans has been questioned recently on the basis of Western blot analyses (9). We found that binding of ^{125}I-TNFα to pooled human IgG was inhibited by excess amounts of unlabeled TNFα. However, papain-treated IgG retained less than 10% of the displaceable binding of ^{125}I-TNFα and more than 50% was recovered by coprecipitation with anti-Fcγ antibodies. Furthermore, skimmed milk suppressed the binding of ^{125}I-TNFα to IgG more strongly than explained by simple, competitive binding of the ligand to milk. Therefore, the occurrence of natural antibodies against TNFα in pooled human IgG and, consequently, a frequent occurrence in normal human serum is questionable.

Different structural forms of human TNFα have been described. For example, x-ray analysis shows that crystalline TNFα is a trimer (43). Furthermore, monomeric, dimeric, trimeric, and higher polymeric forms have been found when examining TNFα in aqueous solution and biological fluids (44, 45). Using molecular size chromatography with recoveries exceeding 95%, we did not observe an increased formation of higher polymers at low concentrations of ^{125}I-TNFα as observed by others (44). However, ^{125}I-TNFα appeared to exist on both a trimeric and monomeric form. The relative distribution between these molecular forms was dependent upon the concentration of the cytokine with a relative dominance of the monomeric form at lower concentrations.

The analyses of the binding of IgG to monomeric as well as trimeric ^{125}I-TNFα suggest that the displaceable binding of ^{125}I-TNFα was caused by a higher nonspecific binding of monomeric ^{125}I-TNFα to the immunoglobulins. Thus, the higher displaceable binding of ^{125}I-TNFα obtained with dimeric IgG may be explained by an increased nonspecific binding of TNFα to this IgG structure.

The results of the present study show the importance of saturation binding analysis and the demonstration of selective binding to Fab fragments when searching for autoantibodies to cytokines. To avoid misinterpretations, the presence of different structural forms of the ligand should be evaluated. Thus, different forms of the cytokine may exhibit different binding to antibodies, as illustrated here by the binding of nondenatured and heat-denatured ^{125}I-IL-1β and monomeric/trimeric ^{125}I-TNFα to IgG.

Reduction of nonspecific binding to antibodies is of obvious importance and may be obtained by adding detergents or, more efficaciously, milk proteins. The latter was the only additive capable of reducing nonspecific binding to IgG of all the ^{125}I-labeled cytokines. However, depending upon the structural form of the cytokine, milk proteins may also reduce the specific binding to antibody. Analysis of ligand binding to milk proteins may therefore help select the proper amount of these proteins for use in assays designed to evaluate specific binding of cytokines to naturally occurring or induced antibodies to cytokines.

IL-1α and IL-6 are biologically active at concentrations below 0.1 ng/ml, and the circulating levels of the cytokines are generally below this concentration (3). However, during gram-negative sepsis or active stages of some immunoinflammatory diseases, blood levels of IL-1α and IL-6 increase considerably; however, the levels of IL-1α and IL-6 rarely exceed 0.5 ng/ml and 2 ng/ml, respectively, although very high levels of IL-6 (up to more than 100 ng/ml) may be seen in patients with septic shock (3, 46–49). Both cytokines are thought to be crucially involved in the pathology of sepsis (3, 4).

Naturally occurring autoantibodies to IL-1α and IL-6 in humans effectively neutralize the bioactivities of these cytokines in vitro (14, 35). A dose of 400 mg/kg per d on 5 consecutive d of the IgG preparations examined in this study would contribute approximately 2 μg of anti-IL-1α IgG and 1 μg of anti-IL-6 IgG per kg body wt. Even though these quantities are 2–3 orders of magnitude lower than those of monoclonal antibodies needed to suppress functions of cytokines in animals (50, 51), the exquisite binding affinities of the autoantibodies would provide a neutralizing capacity similar to that of monoclonals used in animals. For example, Jesmok et al. found significant protective effect of 150 μg/kg of monoclonal antibody to human TNFα in *E. coli*-challenged swine, and 100 ng/ml antibody was required for notable neutralization of TNFα found in the plasma of untreated septic pigs (mean plasma concentration: 2.5 ng/ml of TNFα; reference 51). In comparison, high-dose IgG therapy would contribute about 10 mg/ml to the plasma concentration of IgG; this would provide approximately 10 ng/ml of specific antibody to IL-1α capable of binding 1–2 ng/ml of the cytokine.

It should be noted that IL-1α is predominantly found in the membrane of antigen-presenting cells (52), and because IL-1α as well as IL-6 are bound to surface receptors on responder cells such as lymphocytes (1), very low concentrations of IgG_1 and IgG_2 autoantibodies may trigger complement-mediated cytotoxic processes against these cells. Therefore, the presence of these autoantibodies in pharmaceutically prepared IgG may help to explain why high dose IgG therapy is beneficial in a number of pathogenetically obscure immunoinflammatory disorders.

Acknowledgments

Ms Susanne Meldgaard is thanked for excellent technical assistance. Amersham International generously supplied the radioiodinated cytokines. We are grateful to T. Tsuboi, K. R. Hejnæs, G. R. Adolf, and Amersham Denmark for kind gifts of recombinant human cytokines.

This study was supported by the Danish Blood Donor Foundation, the Danish Rheumatism Association, the Danish Medical Research Council, and the Danish Biotechnology Program.

References

1. Bendtzen, K., M. Svenson, V. Jønsson, and E. Hippe. 1990. Autoantibodies to cytokines—friends or foes? *Immunol. Today.* 11:167–169.

2. Dwyer, J. M. 1992. Manipulating the immune system with immune globulin. *N. Engl. J. Med.* 326:107–116.

3. Dinarello, C. A. 1991. Interleukin-1 and interleukin-1 antagonism. *Blood.* 77:1627–1652.

4. Billiau, A., and F. Vandekerckhove. 1991. Cytokines and their interactions with other inflammatory mediators in the pathogenesis of sepsis and septic shock. *Eur. J. Clin. Invest.* 21:559–573.

5. Jeffes, E. W. B., E. K. Ininns, K. L. Schmitz, R. S. Yamamoto, C. A. Dett, and G. A. Granger. 1989. The presence of antibodies to lymphotoxin and tumor necrosis factor in normal serum. *Arthritis Rheum.* 32:1148–1152.

6. Fomsgaard, A., M. Svenson, and K. Bendtzen. 1989. Auto-antibodies to tumour necrosis factor α in healthy humans and patients with inflammatory diseases and Gram-negative bacterial infections. *Scand. J. Immunol.* 30:219–223.

7. Ross, C., M. B. Hansen, T. Schyberg, and K. Berg. 1990. Autoantibodies to crude human leucocyte interferon (IFN), native human IFN, recombinant human IFN-α2b and human IFN-γ in healthy blood donors. *Clin. Exp. Immunol.* 82:57–62.

8. Caruso, A., C. Bonfanti, D. Colombrita, M. de Francesco, C. de Rango, I. Foresti, F. Gargiulo, R. Gonzales, G. Gribaudo, S. Landolfo, N. Manca, G. Manni, F. Pirali, P. Pollara, G. Ravizzola, G. Scura, L. Terlenghi, E. Viani, and A. Turano. 1990. Natural antibodies to IFN-γ in man and their increase during viral infection. *J. Immunol.* 144:685–690.

9. Leusch, H.-G., G. Sitzler, and S. Markos-Pusztai. 1991. Failure to demonstrate TNFα-specific autoantibodies in human sera by ELISA and Western blot. *J. Immunol. Methods.* 139:145–147.

10. Ikeda, Y., G. Toda, N. Hashimoto, N. Umeda, K. Miyake, M. Yamanaka, and K. Kurokowa. 1991. Naturally occurring anti-interferon-α2a antibodies in patients with acute viral hepatitis. *Clin. Exp. Immunol.* 85:80–84.

11. Figlin, R. A., and L. M. Itri. 1988. Anti-interferon antibodies: A perspective. *Sem. Hematol.* 25(Suppl. 3):9–15.

12. Mae, N., D. J. Liberato, R. Chizzonite, and H. Satoh. 1991. Identification of high-affinity anti-IL-1α autoantibodies in normal human serum as an interfering substance in a sensitive enzyme-linked immunosorbent assay for IL-1α. *Lymphokine Cytokine Res.* 10:61–68.

13. Hansen, M. B., M. Svenson, M. Diamant, and K. Bendtzen. 1991. Anti-interleukin-6 antibodies in normal human serum. *Scand. J. Immunol.* 33:777–781.

14. Hansen, M. B., M. Svenson, M. Diamant, and K. Bendtzen. 1993. High-affinity IgG autoantibodies to IL-6 in sera of normal individuals are competitive inhibitors of IL-6 *in vitro*. *Cytokine.* 5:72–80.

15. Thurmond, L. M., and M. J. Reese. 1991. Immunochemical characterization of human antibodies to lymphoblastoid interferon. *Clin. Exp. Immunol.* 86:514–519.

16. DeForge, L. E., and D. G. Remick. 1991. Sandwich ELISA for detection of picogram quantities of interleukin-8. *Immunol. Invest.* 20:89–97.

17. Mogensen, K. E., P. H. Daubas, I. Gresser, D. Sereni, and B. Varet. 1981. Patient with circulating antibodies to α-interferon. *Lancet.* 2:1227–1228.

18. Bost, K. L., B. H. Hahn, M. S. Saag, G. M. Shaw, D. A. Weigent, and J. E. Blalock. 1988. Individuals infected with HIV possess antibodies against IL-2. *Immunology.* 65:611–615.

19. Svenson, M., L. K. Poulsen, A. Fomsgaard, and K. Bendtzen. 1989. IgG autoantibodies against interleukin 1α in sera of normal individuals. *Scand. J. Immunol.* 29:489–492.

20. Svenson, M., M. B. Hansen, and K. Bendtzen. 1990. Distribution and characterization of autoantibodies to interleukin 1α in normal human sera. *Scand. J. Immunol.* 32:695–701.

21. Saurat, J.-H., J. Schifferli, G. Steiger, J.-M. Dayer, and L. Didierjean. 1991. Anti-interleukin-1α autoantibodies in humans: Characterization, isotype distribution, and receptor-binding inhibition—Higher frequency in Schnitzler's syndrome (urticaria and macroglobulinemia). *J. Allergy Clin. Immunol.* 88:244–256.

22. Suzuki, H., T. Ayabe, J. Kamimura, and H. Kashiwagi. 1991. Anti-IL-1α autoantibodies in patients with rheumatic diseases and in healthy subjects. *Clin. Exp. Immunol.* 85:407–412.

23. Sunder-Plassmann, G., P. L. Sedlacek, R. Sunder-Plassmann, K. Derfler, K. Swoboda, V. Fabrizii, M. M. Hirschl, and P. Balcke. 1991. Anti-interleukin-1α autoantibodies in hemodialysis patients. *Kidney Int.* 40:787–791.

24. Vallbracht, A., J. Treuner, B. Flehmig, K. E. Joester, and D. Niethammer. 1981. Interferon-neutralizing antibodies in a patient treated with human fibroblast interferon. *Nature (Lond.).* 289:496–497.

25. Panem, S., I. J. Check, D. Henriksen, and J. Vilcek. 1982. Antibodies to α-interferon in a patient with systemic lupus erythematosus. *J. Immunol.* 129:1–3.

26. Trown, P. W., M. J. Kramer, R. A. Dennin, E. V. Connell, and A. V. Palleroni. 1983. Antibodies to human leucocyte interferons in cancer patients. *Lancet.* 1:81–87.

27. Suzuki, H., T. Akama, M. Okane, I. Kono, Y. Matsui, K. Yamane, and H. Kashiwagi. 1989. Interleukin-1-inhibitory IgG in sera from some patients with rheumatoid arthritis. *Arthritis Rheum.* 32:1528–1538.

28. Larrick, J. W. 1989. Native interleukin 1 inhibitors. *Immunol. Today.* 10:61–66.

29. Lantz, M., U. Gullberg, E. Nilsson, and I. Olsson. 1990. Characterization in vitro of a human tumor necrosis factor-binding protein. A soluble form of a tumor necrosis factor receptor. *J. Clin. Invest.* 86:1396–1342.

30. Capper, S. J., S. Kalinka, and T. H. Mander. 1990. Specific radioimmunoassays for IL 1α and IL 1β in plasma at physiological and acidic pH: Determination of immunoreactive forms by gel filtration and radioligand binding studies. *Cytokine.* 2:182–189.

31. Hansen, M. B., M. Svenson, and K. Bendtzen. 1992. Serum-induced suppression of interferon (IFN) activity. Lack of evidence for the presence of specific autoantibodies to IFN-α in normal human sera. *Clin. Exp. Immunol.* 88:559–562.

32. Fuhlbrigge, R. C., K. C. F. Sheehan, R. D. Schreiber, D. D. Chaplin, and E. R. Unanue. 1988. Monoclonal antibodies to murine IL-1α. Production, characterization, and inhibition of membrane-associated IL-1 activity. *J. Immunol.* 141:2643–2650.

33. Möller, A., F. Emling, D. Blohm, E. Schlick, and K. Schollmeier. 1990. Monoclonal antibodies to human tumor necrosis factor α: In vitro and in vivo application. *Cytokine.* 2:162–169.

34. Hogquist, K. A., M. A. Nett, K. C. F. Sheehan, K. D. Pendleton, R. D. Schreiber, and D. D. Chaplin. 1991. Generation of monoclonal antibodies to murine IL-1β and demonstration of IL-1 in vivo. *J. Immunol.* 146:1534–1540.

35. Svenson, M., M. B. Hansen, L. Kayser, Å. K. Rasmussen, C. M. Reimert,

and K. Bendtzen. 1992. Effects of human anti-IL-1α autoantibodies on receptor binding and biological activities of IL-1. *Cytokine.* 4:125–133.

36. Morgan, C. D., K. C. Mills, D. L. Lefkowitz, and S. S. Lefkowitz. 1991. An improved colorimetric assay for tumor necrosis factor using WEHI 164 cells cultured on novel microtiter plates. *J. Immunol. Methods.* 145:259–262.

37. Bendtzen, K., M. B. Hansen, M. Diamant, P. Heegaard, and M. Svenson. 1992. Cytokine regulation during inflammation: modulation by autoantibodies and fever. *In* Neuro-Immunology of Fever. T. Bartfai and D. Ottoson, editors. Pergamon Press, Oxford. 215.

38. Tankersley, D. L., M. S. Preston, and J. S. Finlayson. 1988. Immunoglobulin G dimer: an idiotype–anti-idiotype complex. *Mol. Immunol.* 25:41–48.

39. Moonen, P., R. Gaffner, and P. Wingfield. 1988. Native cytokines do not bind to uromodulin (Tamm-Horsfall glycoprotein). *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 226:314–318.

40. Roux, K. H., and D. L. Tankersley. 1990. A view of the human idiotypic repertoire. Electron microscopic and immunologic analyses of spontaneous idiotype-anti-idiotype dimers in pooled human IgG. *J. Immunol.* 144:1387–1395.

41. Harlow, E., and D. Lane. 1988. Antibodies. A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 496 pp.

42. Batteiger, B. E. 1988. Blocking of immunoblots. *In* CRC Handbook of Immunoblotting of Proteins. Vol I. Technical Descriptions. O. J. Bjerrum and N. H. H. Heegaard, editors. CRC Press, Boca Raton, FL, USA. 145.

43. Jones, E. Y., D. I. Stuart, and N. P. C. Walker. 1989. Structure of tumour necrosis factor. *Nature (Lond.).* 338:225–228.

44. Petersen, C. M., A. Nykjær, B. S. Christiansen, L. Heickendorff, S. C. Mogensen, and B. Moller. 1989. Bioactive human recombinant tumor necrosis factor-α: an unstable dimer? *Eur. J. Immunol.* 19:1887–1894.

45. Schoenfeld, H.-J., B. Poeschl, J. R. Frey, H. Loetscher, W. Hunziker, A. Lustig, and M. Zulauf. 1991. Efficient purification of recombinant human tumor necrosis factor β from Escherichia coli yields biologically active protein with a trimeric structure that binds to both tumor necrosis factor receptors. *J. Biol. Chem.* 266:3863–3869.

46. Breen, E. C., A. R. Rezai, K. Nakajima, G. N. Beall, R. T. Mitsuyasu, T. Hirano, T. Kishimoto, and O. Martinez-Maza. 1990. Infection with HIV is associated with elevated IL-6 levels and production. *J. Immunol.* 144:480–484.

47. Eastgate, J. A., J. A. Symons, N. C. Wood, S. J. Capper, and G. W. Duff. 1991. Plasma levels of interleukin-1-alpha in rheumatoid arthritis. *Br. J. Rheumatol.* 30:295–297.

48. Mahida, Y. R., L. Kurlac, A. Gallagher, and C. J. Hawkey. 1991. High circulating concentrations of interleukin-6 in active Crohn's disease but not ulcerative colitis. *Gut.* 32:1531–1534.

49. Damas, P., D. Ledoux, M. Nys, Y. Vrindts, D. De Groote, P. Franchimont, and M. Lamy. 1992. Cytokine serum level during severe sepsis in human IL-6 as a marker of severity. *Ann. Surg.* 215:356–362.

50. Starnes, H. F., Jr., M. K. Pearce, A. Tewari, J. H. Yim, J. C. Zou, and J. S. Abrams. 1990. Anti-IL-6 monoclonal antibodies protect against lethal *Escherichia coli* infection and lethal tumor necrosis factor-α challenge in mice. *J. Immunol.* 145:4185–4191.

51. Jesmok, G., C. Lindsey, M. Duerr, M. Fournel, and T. Emerson, Jr. 1992. Efficacy of monoclonal antibody against human recombinant tumor necrosis factor in *E. coli*-challenged swine. *Am. J. Pathol.* 141:1197–1207.

52. Weaver, C. T., and E. R. Unanue. 1990. The costimulatory function of antigen-presenting cells. *Immunol. Today.* 11:49–55.