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Bioactivities of a tumour necrosis like factor released by chicken rnacrophages

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1. Introduction

Tumour necrosis factor (TNF)-α is a major monocyte/macrophage derived cytokine that can also be produced by many other cell types including lymphocytes, neutrophils. and astrocytes. It is produced in response to invasive stimuli such as bacterial, viral, fungal, parasitic or neoplastic agents. TNF-a induces many gene products involved in inflammation, tissue repair, hematopoesis. immune response, and anti-tumour effects [1]. TNF genes have been cloned in numerous mammalian species [2–10] but attempts to clone chicken TNF have been unsuccessful so far.

The mature form of mammalian TNF-α is a trimer of identical 17 kDa subunits each derived from a 26 kDa initial translation product [11]. TNF induces the production of other gene products such as cytokines and their receptors, adhesion molecules, and class I and II MHC molecules [12–15]. TNF has been reported to synergise with other cytokines, especially with IFN-γ to induce or compound IFN effects [16].

Information about TNF in chickens is limited. Cytotoxic TNF-like activity was shown to be induced by *Eimeria* infection or lipopolysaccharide (LPS) stimulation of chicken macrophage populations [17–20]. TNF-like activity was detected by cytotoxic activity assays performed with mouse [17,18] or chicken fibroblast cell lines [19,20]. Western blotting using anti-human and anti-mouse TNF-α antibodies demonstrated that TNF was expressed during early chick development [21]. However, it is neither known if this factor is the avian homologue of mammalian TNF, nor has this factor been biologically characterised further.

Our objective in this study was to identify an avian TNF-like factor in chicken macrophage cell supernatants and characterise its biological activities in vitro. The production of TNF-like factor was induced by LPS in the chicken macrophage cell line MQ-NCSU [22] and further characterised by ion-exchange and gel permeation chromatography (GPC). Each fraction of MQ-NCSU conditioned medium was tested for the following TNF-like characteristics: cytotoxicity; up-regulation of Ia-expression; NO induction; and synergism with IFN-γ. The cross-reactivity of anti-mammalian TNF antiserum with chicken TNF-like factor was determined, which may give an indication for structural homology between mammalian and chicken TNF.

2. Materials and methods

2.1. Materials

Mouse anti-chicken Ia-FITC was purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL); Leibovitz's L-15 medium, McCoy's 5A medium, RPMI 1640, Dulbecco's modified Eagles medium (DMEM), Penicillin-streptomycin sulfate and L-glutamme were obtained from Sigma (St Louis, MO) as well as LPS, G-75 Sephadex beads, MTT (3-(5,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide and the horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG. Recombinant human TNF-α and rabbit anti-human TNF-a antiserum were obtained from Genzyme Diagnostics (Cambridge, MA): dialysis bags Spectra/Por®, 12-14 kDa cut off, were purchased from Spectrum Medical Industries (Houston, TX); DEAE Sepharose® Fast Flow was purchased from Pharmacia Biotech Products (Piscataway, NJ). Phosphate-buffered saline (PBS) was obtained from Celox Laboratory, Inc. (St Paul, MN); BioBlot-NC®, Nitrocellulose blotting membrane from Costar (Cambridge, MA); ImmunoPure® metal enhanced DAB substrate kit from Pierce Chemical Co. (Rockford, IL); the 10–20% gradient SDS polyacrylamide pre-cast gel, the Mini-protean® II cell and Tween-20 from Bio-Rad (Hercules, CA); and the SeeBlue® Pre-stained standard was purchased from Novex (San Diego, CA).

2.2. Induction of TNF-like activity

To induce TNF-like activity, the chicken macrophage cell line MQ-NCSU [22] was seeded at a cell density of 5×10^5 cells/ml and grown overnight in Leibovitz–McCoy's (1:2) complete medium containing tryptose phosphate broth

(5%). fetal calf serum (FCS; 5%), chicken serum (5%), L-glutamine (200 mM/ml), penicillin (50 U/ml), and streptomycin (50 μg/ml). The medium was replaced by incomplete medium without sera and supplemented with 10 μg/ml LPS. The cells were then stimulated for 16 h, and the supernatant was harvested. The supernatant was clarified by centrifugation at 4000 × g and used for further purification or stored at −80°C.

2.3. Chromatography of TNF-like factor activity

To enrich TNF-like factor activity, one litre of conditioned media was dialysed overnight at 4°C against deionised (DI) water using dialysis bags with a molecular weight cut-off of 12–14 kDa. The dialysed supernatant was concentrated by lyophilisation. The dry lyophilisate was dissolved in 130 ml of buffer 1 (25 mM Tris-base, 50 mM NaCl, pH 7.2) and stored at −90°C until further use. Chromatographical separation of the dissolved lyophilisate was performed on a 1 × 10 cm column of DEAE Sepharose® Fast Flow (FF) at a volumetric flow rate of 1 ml/min. Ten to twenty column volumes (CV) of buffer 1 were utilised to equilibrate the DEAE FF column prior to each run. Forty to eighty millilitres of sample of the reconstituted lyophilisate were loaded to the equilibrated DEAE FF column at a flow rate of 1 ml/min, and the column flow through (FT) was collected. Upon completion of the sample-loading step, the column was washed with 20 CV of buffer 1 until the baseline ABS 280 nm was achieved. The bound proteins were rluted with 10–15 CV of buffer containing 0.4 M and 1.0 M NaCl, pH 7.2.

The FT and the eluates were monitored for protein content at 280 nm absorbency and for TNF activity in the cytotoxicity assay. All chromatographic separations were carried out at 4°C.

The TNF-active fraction from the DEAF-step was concentrated by lyophilisation. The concentrate was then dissolved in 3 ml of column buffer (buffer 2; 20 mM Na_2HPO_4, pH 7.0). Sephadex G-75 beads were swollen in buffer 2 according to manufacturer's instructions. Hundred millilitres of column buffer were run through by gravity ($1 \times g$) to accomplish packing of the column (1.5 × 30 cm). Non-specific binding sites on the resin beads were blocked by including 2 mg/ml bovine serum albumin in buffer 2 during the packing process. A pump and fraction collector were attached to the column and calibrated. Four pools were collected in each of three runs with identical profiles. The peak pools were frozen, lyophilised and resuspended in 15 ml water and dialysed for 4 h at 4°C. The dialysed pools were frozen, relyophilised dry and stored at −80°C. After resuspension in PBS, the samples were monitored for protein absorbency at 280 nm and analysed for TNF activity and for cross-reaction with anti-human TNF-α antibodies in Western blotting procedures.

2.4. Assay for TNF activity

For the assessment of chicken TNF activity, the chicken fibroblast cell line CHCC OU-2 [23] was used in a cytotoxicity assay as the target cell population following previously published procedures [19,24]. Briefly, CHCC OU2 cells were seeded into 96 well plates at 2 x 10^4 cells/well. After overnight incubation of the cells at 41°C, serial diluted samples (100 μl) were added in triplicates to each well. The diluted samples were incubated at 41°C. After 48 h, the plates were spun at 500 × g for 10 min. Twenty five microlitres of PBS containing 2.5 mg/ml MTT were added to each well after one washing step with PBS. Then the cells were lysed after 2 h of incubation at 41°C with 20% SDS in 50% dimethylformamide, pH 4.7, for 1.5 h at room temperature (RT). Measurement of the optical density (OD) of each well was conducted on an automated ELISA reader (Molecular Devices, Menlo Park, CA) at 570 nm. All experimental samples were assayed at least three times and the results combined for data analysis. The specific cytotoxicity was expressed by the following formula: percentage cytotoxicity = (A−B)/(A−C) × 100 where A represents the OD value of control wells containing DMEM alone, B represents the OD of the sample wells, and C represents the absorbency of wells to which 0.5% TritonX100 was added to achieve maximum cy-

tolysis [20]. One unit of TNF is defined as the amount required for 50% cell lysis.

In order to examine the heat stability of chicken TNF-like factor, the samples were incubated at 56°C for 30 min or 95°C for 10 min; in this manner residual LPS-activity could also be excluded prior to the assay [25,26]. In order to examine the antigenic relationship of chicken TNF-like factor with mammalian TNF, the chicken TNF-like factor was neutralised with a polyclonal rabbit anti-human TNF-a antiserum. The samples, in dilutions of 1:100 and 1:1000 were incubated with serial dilutions of the antiserum for 2 h at 37°C. Residual TNF activity of the samples was determined in the cytotoxicity assay. The neutralisation activity of this antiserum was confirmed by using recombinant human (rh) TNF-α under similar condition. Previously published procedures [24] were utilised to test the biological activity of neutralised and non-neutralised rhTNF-α by using L929 cells.

2.5. Bioassay for NO-inducing activity

The NO-inducing activity of chicken TNF-like factor was assayed by using HD11 cells (generously provided by Dr H. S. Lillehoj, United States Department of Agriculture, Washington, MD, USA) following previously published procedures [27]. Briefly, 100 µl of serial dilutions of samples were added to 10^5 HD11 cells cultured in phenol-red-lacking RPMI 1640 medium supplemented with 2% FCS and penicillin/steptomycin (50 U/ml and 50 µg/ml, respectively). After 48 h of incubation at 41°C. 100 µl of these supernatants were assayed for NO using Greiss reagent [27,28]. The results were expressed as U/ml, with 1 U equalling the amount of supernatant required to stimulate 10^5 HD11 cells to produce a concentration of 10 μM of nitrite [27].

In order to determine synergistic effects between chicken TNF-like factors and IFN-y, serial dilutions of Pool 1 or recombinant chicken IFN-y (32 U/well), either alone or combined in 100 µl volume of medium. were added to 10^5 HD11 cells and incubated for 48 h at 41°C. The recombinant chicken IFN-γ was expressed by a recombinant fowlpox virus (FPV) vector in chicken embryo fibroblasts (CEFs) [29] and thr IFN-y-concentration (U/ml) was determined in the VSV-protection assay [30]. As a negative control, supernatant from CEFs infected with the parent FPV was included in the experiment. At the end of the incubation period, supernatants were assayed for NO levels by using Greiss reagent as described previously.

2.6. Flow cytometric analysis of Ia-up-regulation

Cell surface MHC class II antigen expression on MQ-NCSU cells was detected after stimulation with various fractions of chicken TNF-like factor using monoclonal antibodies (Ia) against chicken MHC class II (Ia) [31]. One million cells/ml of adherent MQ-NCSU cells in medium were stimulated with serial dilutions of chicken TNF-like factor for 48 h at 41°C. As a positive control, the cells were stimulated with 10 µg/ml LPS. The cells were scraped off the tissue culture plate, washed once with PBS supplemented with 2% FCS, and 10^6 cells were incubated with mouse anti-chicken Ia-FITC (0.5 µg/10^6 cells) for 1 h on ice. The cells were washed three times in PBS and fixed with 4% paraformaldehyde. A FACSCalibur (Becton Dickinson, San Jose, CA) was used to analyse ten thousand cells per sample (FL-I channel).

2.7. SDS–polyacrylamide gel electrophoresis and (PAGE) western blotting

The samples were diluted 1:10 in DI water followed by addition of the same volume of denaturing sample buffer (0.125 M Tris, 4% SDS, 20% Glycerol, 0.2 M DTT, 0.02% bromophenol blue). The samples were heated to 95°C for 5 min and 20 pl were loaded onto each lane of a 10–20% SDS polyacrylamide gel and electrophoresed for 1.5 h at 100 V.

At this point the proteins were transferred at 4°C from the gel to a nitrocellulose membrane overnight at 200 mA constant current using a Mini-protean® II cell. Nonspecific binding of immunoglobulin was blocked by treating the transfer membrane with PBS containing 0.5% nonfat milk for 4 h at RT. After three washing steps with PBS containing 0.05% Tween-20 (PBS-T) for 10 min each, the membranes were incubated overnight at 4°C with rabbit-anti-human TNF-α (1:1000), or pre-immune rabbit serum (1:1000) in PBS-T. This was followed by three washings with PBS-T and 1 h incubation with HRP-labelled goat-anti-rabbit immunoglobulin (1:100) in PBS-T. After three washing steps with PBS-T for 10 min each, the blots were developed with ImmunoPure® containing DAB for 5 min. The molecular weight was estimated by using the molecular weight standards SeeBlue® electrophoresed on the same gel.

Table 1
Purification of chicken TNF-like factor from cell culture supernatant of LPS-stimulated MQ-NCSU cells

Purification step	Final volume (ml)	Total protein (mg)	Total cytotoxic activity (U)	Specific activity (U/mg)	Purification factor[a] of TNF-like activity
Starting material	1000	2700	2×10^5	0.07×10^3	
DEAE-Sepharose FT-fraction	3	61	0.14×10^5	0.2×10^3	2.9
G-75 Sephadex					
Pool 1	1	0.5	0.08×10^5	6.1×10^3	87.0
Pool2	1	5.1	0.03×10^5	0.6×10^3	8.5
Pool 3	0.5	2.6	0.02×10^3	0.58×10^3	8.2
Pool 4	1.5	6.6	0.018×10^3	0.27×10^3	3.8

[a] Purification factor, specific activity of starting material/specific activity of the collected fractions.

3. Results

3.1. Characterisation of the cytotoxic activity of chicken TNF-like factor

The chicken macrophage cell line MQ-NCSU was cultured in serum-free medium for 16 h in the presence of 10 μg/ml LPS. Under these conditions, this cell line produced about 200 U TNF-like activity per millilitre as estimated by the cytotoxicity assay. This amount is equivalent to a specific activity of 70 U/mg protein (Table 1). The FT fraction contained greater than 90% of the TNF activity in comparison to the fractions eluted at 0.4 M and 1.0 M NaCl, which contained 5–10% of the recovered activity (data not shown).

Fig. 1 shows a typical chromatography profile obtained from the fractionation of the pooled FT fractions on the G-75 GPC column. The pools represent in order, the void peak (Pool 1: > 81 kDa), a shoulder of the void peak (Pool 2: 70–55 kDa), a transition (Pool 3: 40–35 kDa) and a small included peak (Pool 4: < 25 kDa) (Fig. 1). Pool 1 had the highest specific TNF-like activity with 6.1×10^3 U/mg recovered protein; Pools 2, 3 and 4 had cytotoxic activities of 0.6×10^3 U/mg, 0.58×10^3 U/mg, and 0.27×10^3 U/mg, respectively (Table 1). The dose response curve for Pool 1 demonstrates a clear reduction of TNF-like activity with increasing dilutions (Fig. 2). Eighty percent of the TNF-like activity remained after treatment of Pool 1 at 56°C for

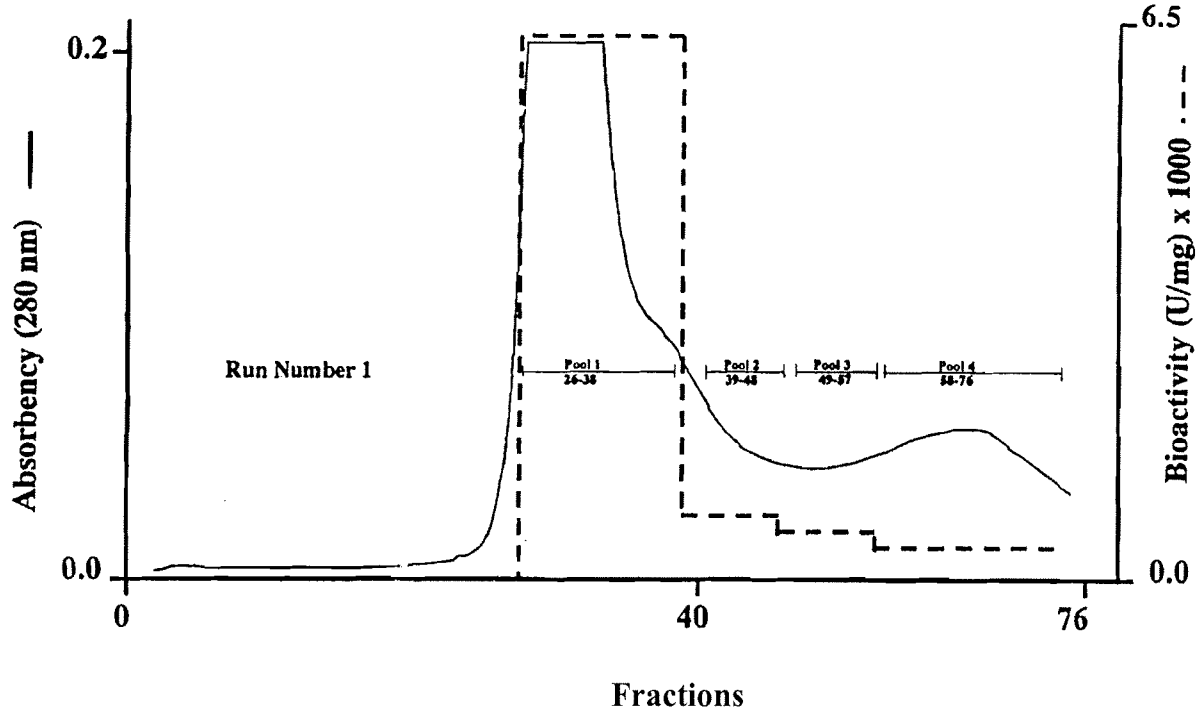

Fig. 1. G-75 Sephadex gel-permeation chromatography. The column was equilibrated with buffer 2. The pump flow rate was adjusted to 30 ml/h. Fractions were measured by drops and were set at 38 drops per fraction which equals 1 ml. Using blue dextran (MW 2,000,000) and phenol red (MW 345), the column was calibrated. Blue dextran marks the V_0 (void volume) and phenol red marks the V, (total volume) of the column. Per run, 1 ml of sample was loaded onto the column by gravity and fractionation was started immediately. Three runs with identical profiles were performed with 1 ml of sample (4.7×10^5 U of cytotoxic activity/1 ml). Four pools were collected in each run. The pools represent in order the void peak (pool 1: > 81 kDa); a shoulder of the void peak (pool *2: 70–55* kDa); a transition (pool 3: 40–35 kDa); and a small included peak (pool 4: < *25* kDa). Pools *1–4* were combined based on the protein profiles.

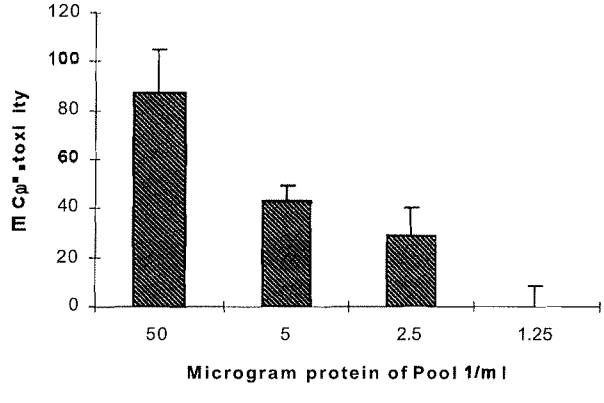

Fig. 2. Dose response of TNF-like factor (Pool 1) in the cytotoxicity assay.

30 min, suggesting heat stability of chicken TNF-like factor (data not shown) [25,26]. No cytotoxic activity remained after heating the sample to 95°C (data not shown). The biological activity of chicken TNF-like factor was not neutralised by the neutralising rabbit-anti-human TNF-α antiserum (data not shown).

3.2. Macrophage stimulation by chicken TNF-like factor

TNF has a potent activating effect on macrophages in mammals. Fig. 3b shows that Pool 1 induced morphological changes in the chicken macrophage cells MQ-NCSU. The cellular granularity was increased and the number of cellular

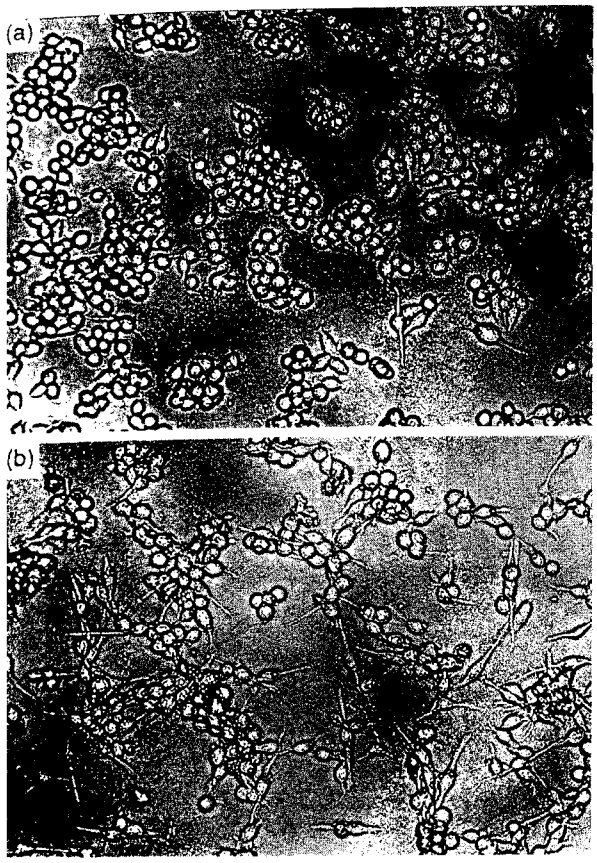

Fig. 3. Morphological changes induced in MQ-NCSU cells. (a) Non-stimulated MQ-NCSU cells after 48 h of culture in growth medium (×400). Less than 20% of the cells show cellular extensions and cytoplasmic granules. (b) MQ-NCSU cells after 48 h of stimulation with growth medium supplemented with 12 µg of pool 1 1 × 10^6 cells (×400). Over 80%, of the cells show long cellular extensions and granules.

extensions was enhanced after stimulation with Pool 1 in comparison to the medium control (Table 2, Fig. 3b). Furthermore, Pool 1 enhanced Ia-expression by MQ-NCSU cells by 2.38 fold and induced NO production by HD11 cells (Table 7, Fig. 4). No NO inducing activity remained after heating the sample to 95°C (data not shown).

Pool 2 had strong effects on NO induction and enhanced Ia-expression 1.8 fold (Table *2*). Pool *3* showed NO inducing activity but had no effect on Ia expression by MQ-NCSU cells (Table 2). Pool 4 had residual effects on macrophage activation, induced morphological changes, and enhanced Ia expression about 1.16 fold (Table 2).

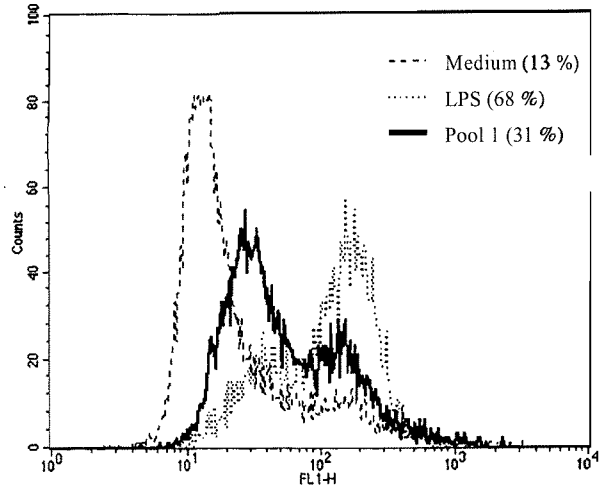

Fig. 4. Flow cytometric analysis of Ia-expression by MQ-NCSU cells after culturing with medium, LPS (10 µg/ml) or Pool 1 (1:200) for 48 h. The percentage of cells positive for Ia-expression is presented in parenthesis.

These findings indicate that Pool 1 was positive for all the TNF-like biological activities we tested for. Pool 1 was used further to study synergistic effects of chicken TNF-like factor with IFN-γ. In previous studies it was demonstrated that mammalian TNF had synergistic effects with IFN-γ on NO induction [32]. Fig. 5 shows that chicken TNF-like factor had synergistic effects with chicken recombinant IFN-γ on the induction of NO production by the chicken macrophage cell line HD11. While *32* U/ml of IFN-γ alone only induced 18 µM of NO, and chicken TNF-like factor at a dilution of 1:400 induced 24 µM of NO or less. the combination of both cytokines enhanced NO production about three times.

3.3. Western blot analysis of avian *TNF-like factor*

Fig. 6 shows a Western blot analysis of Pool 1 and the FT fraction obtained from the ion-exchange step. Lane 1 shows a 20 ng application of rhTNF. The rabbit polyclonal anti-human TNF-α antiserum recognised a strong 17 kDa protein band, possibly the TNF-monomer, and a

Table 2
Macrophage activation by pools collected by GPC from LPS-stimulated MQ-NCSU cells (The results of two repeat experiment are presented.)

Pool[a]	Induction of morphological changes	Time fold increase in la-expression	NO inducing factor (U/mg
1	+++[b]	2.38[c]	6080 ± 480[d,1]
2	–	1.8	760.8 ± 7.2[2]
3	–	0	876.9 ± 61.5[2]
4	++	1.16	0 ± 30.3[3]

[a] Pool 1. >81 kDa molecular weight proteins; pool 2, 70–55 kDa; pool 3, 40–35 kDa; pool 4, <25 kDa.

[b] +++. >90% of MQ-NCSU cells showed one or more cell-extensions and enhanced granularity after stimulation in compari son to non-stimulated control cell cultures; ++, 80–50% of MQ-NCSU cells showed one or more cell-extensions; +. 50–20% o MQ-NCSU cells showed cell-extensions; –, <20% of MQ-NCSU cells showed cell-extensions.

[c] MQ-NCSU cells were stimulated for 48 h with several fractions diluted 1:200 in medium. The cells were harvested and stainec for Ia-cell surface expression. The intensity of Ia expression was determined by flow cytometric analysis. 12 ± 0.6% of unstimulatec MQ-NCSU cells expressed Ia.

[d] Average of two repeat experiments ± standard deviation. Numbers with different superscript figures in one column are signifi cantly different ($P < 0.05$).

weak band at 38 kDa. Lane 2 shows that the rabbit anti-human TNF antiserum recognised also a 17 kDa protein in Pool 1. The antiserum detected additional proteins of 82 kDa in Pool 1. Preimmune rabbit serum did not detect any protein bands in either of the tested samples (Pool 1 see lane 4 Fig. 6). Lane 3 shows that the polyclonal antiserum against rh TNF also recognised proteins of 82 kDa in the FT fraction. No specific protein bands were detected by the polyclonal rabbit anti-rh TNF antiserum in Pools 2–4 (data not shown).

4. Discussion

This study demonstrated for the first time that chicken TNF-like factor has biological activitier homologous to mammalian TNF. Previous studies have indicated that chicken cells produce TNF following LPS or *Eimeria* stimulation [17,19,20,33]. The TNF was identified by a cytotoxicity assay or with cross-reacting polyclonal anti-mammalian TNF-antibodies [17,19,21]. However, these detection methods were not standardised for the chicken system and therefore dc

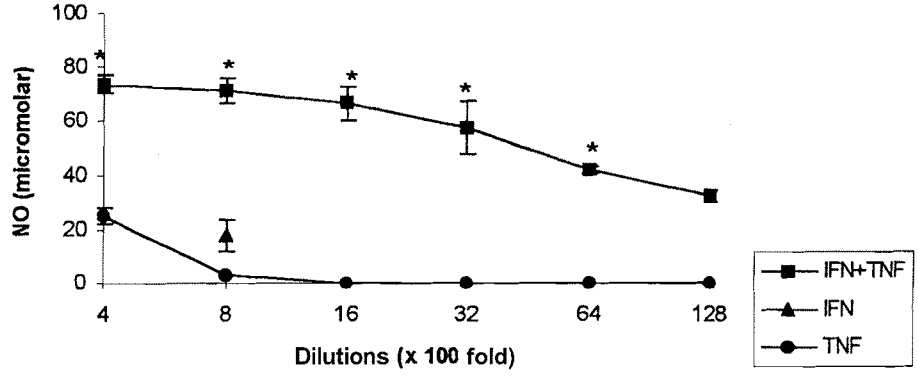

Fig. 5. Synergistic induction of NO production in HD11 cells by serial dilutions of Pool 1 and chicken recombinant IFN-y (*32* U/ ml). The combined data of two repeat experiments are presented. The concentration of NO in non-stimulated or cultures stimulated with supernatant from CEFs infected with FPV was < 1 μM. TNF, chicken TNF-like factor: IFN, IFN-y. 'Significantly different than NO concentrations after stimulation with IFN-y or TNF-like factor alone ($P < 0.05$).

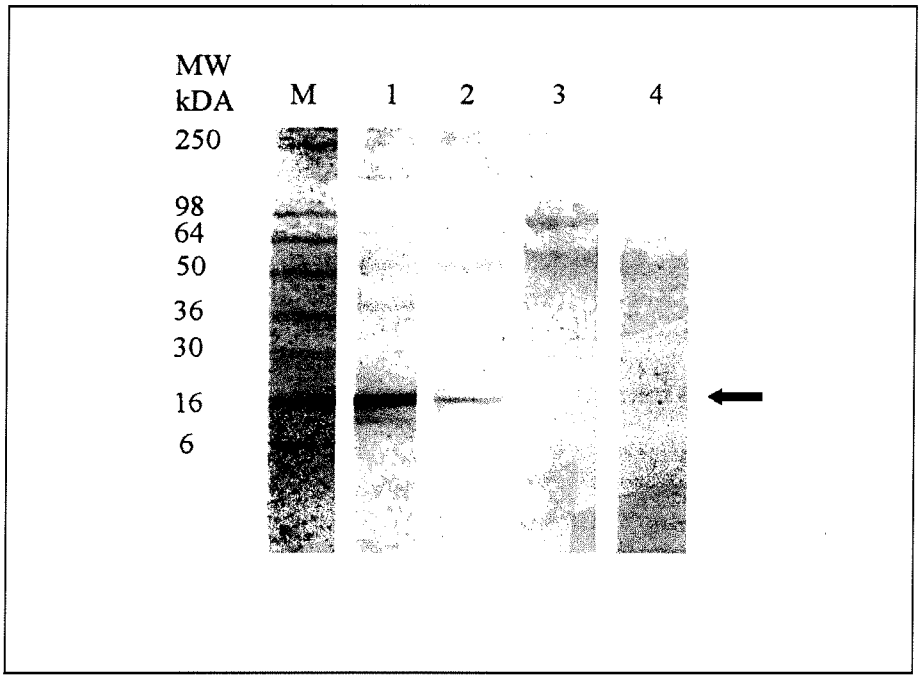

Fig. 6. Western blot of chicken TNF-like factor. SDS–PAGE-separated proteins were transblotted to a Imrnobilon-P transfer membrane then stained with rabbit polyclonal antibodies against recombinant human (rh) TNF-α (lanes 1–3), and developed with the prrouidase-system. Lane M: pre-stained molecular weight marker; lane 1: 20 ng rh TNF: lane 2: 1 μg of Pool 1; lane 3: 40 μg of FT fraction. Lane 4: 1 μg of Pool 1 stained with a 1:500 dilution of pre-immune rabbit serum. Arrow indicates the 17 kDa protein band of similar size to human TNF-α monomers.

not reflect specificity. The lack of specific monoclonal antibodies to identify chicken TNF makes it necessary to characterise chicken TNF by more than one procedure.

Macrophages have been shown to release an impressive panel of cytokines after stimulation such as IL-1, IL-1ra, IL-6, IL-8, IL-10, IL-12, TNF-α, IFN-a, IFN-γ, MCP-1, MCP-3, MIF, M-CSF, G-CSF, GM-CSF, MIP-1, MIP-2, LIF, OSM, and TGF-β [34]. They rapidly synthesise de novo mRNA and protein following activation. Most of these cytokines show similar molecular size and overlapping activities. To more specifically characterise chicken TNF, conditioned medium from LPS-stimulated macrophages was semi-purified by chromatographical methods and tested for TNF-like activity in a variety of activity assays.

By dialysing the supernatants from LPS-stimulated MQ-NCSU cells we separated molecules smaller than 12–14 kDa from our starting material. This procedure excluded chemokine monomers, which have a molecular weight of 8–10 kDa [35] and may have overlapping functions with TNF [41] from our material. After two chromatography steps, most of the chicken TNF-like activity was recovered in the fractions containing proteins above 81 kDa molecular weight. Some residual or additional activity was detected in other fractions, which may be due to other factors of different molecular weight having overlapping function with chicken TNF-like factor. Chicken TNF-like factor is likely to be a peptide multimer in its biologically most active form as shown for mammalian TNF [11]. Residual activity in pools containing proteins of lower molecular weight than 81 kDa may be due to TNF-monomers or dimers. The Western blotting results support this speculation. After denaturation of the proteins from Pool 1, proteins of

17 kDa were detected with polyclonal anti-human TNF antiserum. These proteins had a molecular weight homologous to the human TNF-α monomers. This finding coincides with that of Zhang et al. [19] who detected a 17 kDa band in culture supernatant of blood leukocyte-derived macrophages stimulated with *Eimeria* sporozoites by using a polyclonal anti-human TNF-a serum. We also detected a weak band of proteins of 82 kDa in Pool 1 and the FT fraction, cross-reacting with the polyclonal rabbit anti-human TNF-α antiserum. These may be multimer complexes of TNF not dissociated by the denaturing process.

Polyclonal anti-human TNF-a antiserum did not neutralise the biological activity of chicken TNF-like factor in vitro. This result is not unexpected because homology between chicken and mammalian cytokines cloned so far is relatively low on the protein level [36]. For example, chicken type I IFN has less than 30% homology to mammalian IFN on the amino acid level [30]. Type II IFN has less than 36% homology on the amino acid level to mammalian type II IFN [37].

As demonstrated by the Western blotting procedures, anti-TNF-a serum detects denatured chicken TNF-like factor monomers of 17 kDa possibly due to the recognition of linear epitopes of TNF. The lack of neutralisation of the TNF-like bioactivity by the polyclonal anti-human TNF-a antiserum indicated that the homology of mammalian and chicken TNF at the native protein level may be very low. This may be a reason why attempts to clone chicken TNF on the basis of known mammalian TNF cDNA sequences failed.

Other studies indicated that polyclonal anti-human TNF-a antiserum neutralised chicken TNF-like activity partially in certain chicken strains in vivo [19]. Why anti-human TNF-a antiserum neutralised chicken TNF-like activity partially in vivo but not in vitro is not known.

To distinguish TNF-activity from biological activities of other cytokine in the fractionated supernatants of LPS-stimulated macrophages. we chose to test for a variety of biological functions characteristic of mammalian TNF. TNF is a very potent immunomodulator and stimulator of macrophage function. Major activities incluc the enhancement of the expression of MHC ger products and adhesion molecules [38]. Witsc and Schook [39] showed a feedback control c monocgtic differentiation by TNF. TNF als induced chemotactic migration in human mond cytes at very low concentrations [40]. This stud demonstrated that chicken TNF is a macrophag stimulator. Pool 1 up-regulated MHC class II ex pression 2.38 fold and induced morphologica changes of macrophages. Pool 4 also showe some activity on the induction of morphologica changes. The effect of Pool 4 might be due t other factors than TNF or to monomers of TNI having some residual activity on macrophage ac tivation.

NO production is also an indication fo macrophage activation. The highest NO-inducing effect was shown after stimulation of HDI1 cells with Pool 1. Some activity was also detected ir Pools 2 and 3. It is possible that our separation protocol isolated different NO-inducing factors in Pools 2 and 3 that have not been characterised yet.

Chicken TNF synergised with IFN-y in the induction of NO production by macrophages. Similar observations were made in in vitro models with mammalian cells [41]. It was noted that IFN increased NO production and enhanced the cytotoxic effect of TNF on tumour cells [42,43].

Similar to TNF, IL-1 is also produced by activated mononuclear phagocytes. It has several overlapping functions with TNF. The biologically active forms of IL-1 are monomers of approximately 17 kDa [44]. By fractionating the supernatant of LPS-stimulated MQ-NCSU cells, we were able to exclude the possibility that we purified IL-1 instead of TNF, because the major activity was recovered in Pool 1 that contained proteins above 81 kDa molecular weight. Although IL-1 shares extensive similarities with TNF-actions such as inflammatory and pro-coagulant properties, it does not increase MHC expression in cells [45]. In this study it was demonstrated that factors in Pool 1 up-regulated the expression of MHC class II on chicken macrophages. Based on these observations we

concluded that we did not collect chicken IL-1 in Pool 1.

For the first time it was shown that chicken TNF has several functions homologous to mammalian TNF-a. Chicken TNF had cytotoxic activity for chicken fibroblast cells, induced NO production, and synergised with recombinant chicken IFN-γ. Chicken TNF enhanced Ia expression on MQ-NCSU macrophage cells and induced morphological changes in these cells. Chicken TNF may be a protein complex of above 81 kDa in its active form. Based on these observations, we conclude that chicken macrophages indeed secrete a TNF-like factor, comparable in its activities with mammalian TNF-a.

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