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Tumour necrosis factor in synovial exudates

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SUMMARY The actions of tumour necrosis factor (TNF) include resorption of bone and cartilage suggesting a potential role in the pathogenesis of arthritis. TNF activity was looked for in synovial fluids from 137 patients with different rheumatic diseases. Unfractionated samples were tested in the L929 bioassay. Significant TNF activity that was neutralised by monoclonal antibody to TNF alpha occurred in 13 (30%) of 44 samples. Raised TNF levels were not associated with any particular disease type or routine laboratory markers of inflammation but were related to disease duration in osteoarthritis. The finding of biologically active TNF in symptomatic joints of arthritic patients supports the idea that it may contribute to the pathogenesis of joint damage in chronic rheumatic diseases.

Key word: arthritis.

Cytokines are inducible peptides with receptor mediated biological actions on many different cellular targets. Cytokine production has mainly been associated with leucocyte or fibroblastoid cells during responses to injury or infection.[1 2] Tumour necrosis factors (TNFs) are cytokines that have recently been characterised at molecular level.[3 4] TNF alpha (also called cachectin) is a 17 000 Mr peptide that forms biologically active oligomers.[5] Mononuclear phagocytes are potent sources of TNF alpha,[6] but T cells also produce a similar molecule[7] as well as the related product, lymphotoxin (also called TNF beta).[8] Resting cells release little or no TNF alpha, but after activation mRNA accumulates and TNF peptide appears soon after.[9] The biological activities of human TNF alpha are similar to those of interleukin 1 (IL1) alpha and beta except that human TNF does not activate murine T cells.[10]

The biological role of TNF has not yet been completely defined, but its properties in vitro and in vivo suggest it could be an important mediator of host defence against tumours and pathogens.[11] It is also likely that TNF may contribute to autoimmune and inflammatory pathology such as occurs in rheumatic diseases. The defined activities of TNF relevant to arthritis include resorption of cartilage and bone[12 13]; endothelial adherence and activation of granulocytes[14 15]; stimulation of fibroblast growth[16]; stimulation of synovial cell prostaglandin and collagenase release[17]; and the systemic reactions of fever,[18] liver acute phase protein synthesis,[19] and catabolism of protein and fat leading to muscle loss and cachexia.[20] Whether resident cells in healthy skeletal tissues include populations that produce TNF is not known, but the mononuclear infiltrating cells that characterise synovial pathology in many arthritic diseases are likely sources of TNF. Cytokine studies in arthritis have previously concentrated on interleukins 1 and 2 and interferons,[21] but the connective tissue effects of TNF raise the possibility that it may mediate bone and cartilage injury in chronic joint disease.[20]

As there was no previous information on the occurrence of TNF in human arthritic diseases we tested unfractionated synovial exudate fluids from 137 patients with different rheumatic diseases and report here that biologically significant levels of TNF alpha were found in approximately 30% of the samples.

Materials and methods

SYNOVIAL FLUIDS

Synovial effusion fluids (SF) were obtained from patients referred for aspiration of symptomatic knee joints. Clinical diagnoses covered a wide range of rheumatic diseases as indicated in the results section. All of the patients were being treated with non-steroidal anti-inflammatory drugs and some

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received slow acting anti-inflammatory drugs or low dose oral corticosteroids. Fluids were collected in sterile containers in the presence of 5×10^{-3} M edetic acid, centrifuged at 1800 *g* for 30 minutes, and sample aliquots stored at −70°C or tested immediately for TNF content. The fluids were collected over 16 months.

REAGENTS

Human recombinant (HR) TNF alpha, human recombinant lymphotoxin (TNF beta), mouse monoclonal antibody specific for human TNF alpha and rabbit antiserum against HR lymphotoxin were all kindly donated by Dr Gunther Adolf (Ernst-Boehringer-Institut fur Arzneimittel-Forschung, A-1121 Wien, Austria). HR IL1 alpha and IL1 beta were obtained from Biogen (Geneva). Reagents for immunoradiometric assay for human interferon gamma were from Boots-Celltech (Slough, Berkshire).

TNF ASSAY

The conventional bioassay for TNF activity based on cytotoxicity for the mouse connective tissue cell line L929 was used.[22] Briefly, L929 cells were cultured in Eagle's minimum essential medium (Sigma, St Louis, MO) with 5% fetal calf serum (Gibco, Paisley, UK) at 4×10^4 cells/well for 20 hours in flat bottomed, 96 well, microtitre plates. The medium above the non-confluent cell layer was replaced with fresh medium containing actinomycin D (1 μg/ml) and serial dilutions of standard HR TNF alpha or samples to be tested for TNF content. After a further 20 hour incubation the medium was removed and a 0·5% in 20% methanol solution of crystal violet was added to the wells. The number of viable (adherent) cells remaining in each well was assessed by scanning spectrophotometry (Dynatech MR 700) at 540 nm. The concentration of TNF giving 50% maximal cell killing (one bioassay unit) was equivalent approximately to 50 pg/ml of recombinant human TNF alpha. A range of cytokines, including recombinant and purified natural IL1 alpha and beta, purified interferon alpha and recombinant interferon gamma, and crude interleukins 2 and 3, gave no appreciable activity (less than 4 units/ml) in this assay but HR lymphotoxin was equipotent with TNF alpha.

Synergy between interferon gamma and TNF has been reported for human cell cytotoxicity[23] and in other human systems.[24] Therefore we measured interferon gamma levels in these synovial exudates. Of 27 SF tested for IFN gamma in a specific radioimmunometric assay, none reached the detection limit (1 U/ml). With this assay there was no loss of detection of HR interferon gamma added to osteoarthritis or rheumatoid arthritis synovial fluids compared with culture medium.

Ninety three SF were tested at 11 serial dilutions in at least three separate assays, from which quantitative data (units of TNF activity) could be derived.

SPECIFICITY TESTING

To test that the L929 cytotoxicity of the SF was mediated by TNF alpha some positive samples were reassayed in the presence of monoclonal antibody (MAb) specific for HR TNF alpha or MAb specific for human major histocompatibility complex (MHC) class II product (control antibody). These quantitative experiments indicated that some cytotoxic samples were not neutralised by anti-TNF alpha. To determine the proportion of fluids with cytotoxic activity that were neutralised by anti-TNF alpha we tested a further 44 fresh fluids at one dilution only (1:8) after preincubation with culture medium, anti-TNF alpha, irrelevant MAb, or a rabbit antiserum against HR lymphotoxin.

It is known that both IL1 alpha (di Giovine, Poole, and Duff, unpublished observation) and beta[25] are detectable by radioimmunoassay in human synovial exudate fluids. IL1 is reported to act synergistically with TNF in a number of biological systems.[26–28] To test whether our bioassay estimate of TNF content might be influenced by synovial fluid IL1 we performed L929 bioassays of HR TNF in the presence of HR IL1 alpha and beta. Fig. 1 shows that IL1 did not affect the L929 response to TNF alpha.

Results

TNF-LIKE ACTIVITY IN SYNOVIAL FLUIDS

Of the first 93 SF tested, 35 had TNF-like activity of 4 U/ml or greater. Table 1 summarises the clinical diagnosis and level of TNF-like activity. The two positive fluids in the miscellaneous group were from patients with recurrent monarthritis. Several SF with the highest levels had been assayed fresh, suggesting that activity may be lost after freeze-thawing or storage at −70°C.

ANTIBODY NEUTRALISATION OF TNF-LIKE ACTIVITY

Eight of the 35 fluids with TNF-like activity were retested in full titrations alone or with anti-TNF alpha, or control MAb (Table 2). Six were completely neutralised by anti-TNF alpha but two were not, suggesting that other cytotoxins were present in these samples. To determine the proportion of SF that contained cytotoxic activity attributable to TNF alpha we tested a new group of 44 SF in triplicate at

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one dilution only, either alone or with antibodies. Of these 44 fluids, 13 (30%) contained TNF activity that was unaffected by control antibody but completely neutralised with anti-TNF. They included SF from patients with rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, psoriatic arthritis, reactive arthritis, septic arthritis, and gout. SF that did not contain TNF activity were also found from patients in most of these disease groups. Twenty three samples (52%) contained no TNF-like activity at all and eight (18%) were cytotoxic for L929 cells but either were not neutralised with anti-TNF or were affected by the irrelevant MAb. Notably, of the 21 SF that were cytotoxic for L929, none was neutralised by antiserum to lymphotoxin. This antiserum at 1:800 dilution completely inhibited the activity of 10 ng/ml human recombinant lymphotoxin in the L929 assay.

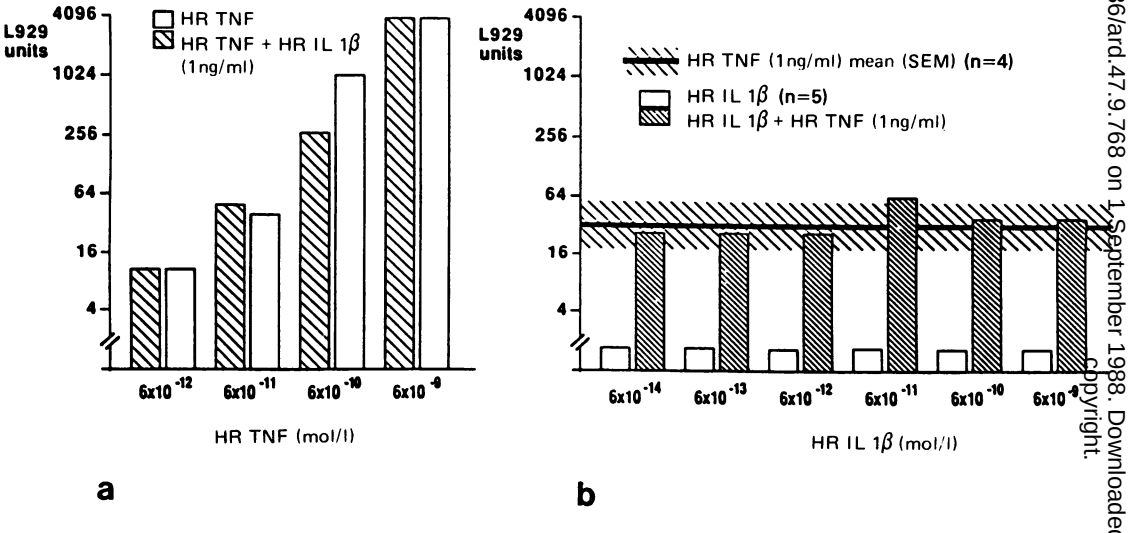

Fig. 1 *Human recombinant tumour necrosis factor (HR TNF) alpha and HR interleukin 1 beta (IL 1β) were tested for synergy in the L929 cytotoxicity assay. (a) Different concentrations of HR TNF alpha were tested in the presence or absence of HR IL1 beta (1 ng/ml). (b) HR TNF alpha (1 ng/ml) was tested in the presence or absence of different concentrations of HR IL1 beta. No synergy in L929 cytotoxicity was observed and HR IL1 beta alone in a wide concentration range was inactive in this assay. Identical results (data not shown) were obtained in separate experiments in which TNF was tested at higher concentrations (10 ng/ml) or HR IL1 alpha was used. Biological activity of both IL1 preparations was confirmed by EL4.NOB.1/CTLL conversion assay.*

Table 1 *TNF activity in SF from patients with different rheumatic diseases*

TNF activity (U/ml)*	*RA+*	*RA−*	*OA*	*ReA*	*PsA*	*AS*	*Misc*	*Total*
<4	29†	11	6	2	3	1	6	58
4–16	9	7	3	4	2	1	1	27
16–28	2		1	1		1		5
28–40			1					1
40–52	1							1
>52							1	1
Total	41	18	11	7	5	3	8	93
% Positive‡	29	39	46	71	40	67	25	38

RA+=seropositive rheumatoid arthritis; RA−=seronegative rheumatoid arthritis; OA=osteoarthritis; ReA=reactive arthritis; PsA=psoriatic arthritis; AS=ankylosing spondylitis; Misc=miscellaneous group.
*Each synovial fluid was measured at least three times at 11 dilutions in different assays.
†Number of patient SF in this category.
‡Percentage of patients in each category with TNF levels> 4 U/ml (detection limit).

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Table 2 *Antibody neutralisation of synovial fluid TNF-like activity*

	Sample alone	*Sample with anti-TNF†*	*Sample with anti-DR‡*
SF 73 (OA)	39*	<4	34
SF 69 (AS)	42	<4	41
SF 78 (OA)	14	<4	ND§
SF 158 (OA)	11	<4	11
SF 161 (RA+)	50	<4	49
SF 91 (RA+)	5	<4	ND
SF 116 (RA+)	19	5	10
SF 112 (ReA)	7	9	ND
HR LT (1 ng/ml)§	24	22	30
HR TNF (1 ng/ml)	22	<4	21

*Values are TNF bioassay units/ml.
†Monoclonal antibody against human recombinant TNF alpha (1:1600).
‡Monoclonal antibody against human MHC class II product as control antibody (1:800).
§HR LT=human recombinant lymphotoxin; ND=not done; other abbreviations as in Table 1.

Discussion

The purpose of this study was to test whether unfractionated synovial exudate fluids from arthritic patients contained biologically active TNF. Using a bioassay for TNF based on cytotoxicity for the murine L929 connective tissue cell line we tested a total of 137 synovial exudate fluids. In quantitative assays for TNF activity 35 (38%) of 93 samples were positive at a concentration of at least 4 U/ml (the limit of detection). In six of eight positive SF tested, TNF-like activity was neutralised by monoclonal anti-TNF alpha. Of a further 44 fluids that were tested in a single dilution assay, 13 (30%) contained TNF activity neutralised only by MAb for TNF alpha. None of the 21 cytotoxic samples in this series was neutralised by antiserum to lymphotoxin.

TNF alpha was found in SF from patients with a variety of rheumatic diseases, but in this preliminary study we noted no association between synovial fluid TNF level and disease category, drug treatment, or laboratory data (haemoglobin, white cells, platelets, erythrocyte sedimentation rate). The only significant difference in clinical terms was in disease duration in osteoarthritis and reactive arthritis, where those with detectable TNF levels had a mean duration of disease of 9·3 years (n=10), while similar patients with no detectable TNF had mean disease duration of 3·3 years (n=14, p=0·0067). Presumably, systemic markers of inflammation are more likely to be related to levels of TNF in the circulation rather than in the synovial space.

There have been recent reports of TNF in samples from patients with meningococcal[29] or protozoal[30] infections and in cancer,[31] but no previous reports of TNF in sterile inflammatory diseases seem to be available. Our results indicate that about 30% of synovial exudates from different rheumatic diseases contain biologically detectable levels of TNF alpha (equivalent to nanomolar concentrations of HR TNF). The presence of TNF in synovial exudate from several rheumatic diseases argues against TNF production as a specific factor in the pathogenesis of any particular disease. It seems that TNF may be associated with the process of joint effusion that is common to a number of articular disorders with apparently different aetiologies. This is in keeping with the known variety of stimuli able to induce TNF production in mononuclear cell populations. For example, monosodium urate crystals stimulate monocyte TNF release in vitro,[32] which could account for TNF in gouty effusions. Presumably, in septic arthritis macrophage TNF production is directly stimulated by microbial products, and TNF release could be a direct or indirect result of lymphocyte activation in both acute and chronic rheumatic diseases. It is likely that these biodetection results underestimate the amount of TNF present as in some fluids we could detect only 50–60% of known quantities of added HR TNF alpha. It is also possible that TNF activity was lost on prolonged storage or during freeze-thawing as the highest levels we found were in fresh or recently collected samples. Finally, the assay itself is relatively insensitive and preliminary findings with more sensitive immunoassays indicate that TNF is detectable in a much larger proportion of samples.

As TNF is known to activate polymorphonuclear cells,[14 15] stimulate synovial prostaglandin production,[17] and increase bone[13 33] and cartilage[12] destruction in vitro, its presence in synovial effusion fluids may well be significant. Recently, TNF has also been shown to induce other cytokines, including IL1 and granulocyte-monocyte colony stimulating factor.[18 34] IL1 is, like TNF, proinflammatory and catabolic[21] and is found in human synovial exudates.[25 35 36] It is made by synovial cells[37 38] and was reported to be arthritogenic when injected into rabbit joints.[39] In addition to IL1 induction by TNF, IL1 itself has been shown to increase TNF activity,[40] suggesting a mechanism for the maintenance of inflammation.

This report now provides evidence that TNF alpha in a biologically active form occurs at the site of inflammation in several types of arthritis and supports the idea that mononuclear cytokines mediate joint tissue destruction.[41] An understanding of the cellular origins and activators of these cytokines should help elucidate the pathogenesis of several types of rheumatic disease.

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