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Regulation of Cytokines, Cytokine Inhibitors, and Acute-Phase Proteins Following Anti-TNF-α Therapy in Rheumatoid Arthritis[1]

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Treatment with a chimeric mAb to TNF-α has been shown to suppress inflammation and improve patient well-being in rheumatoid arthritis (RA), but the mechanisms of action of such treatment have not been fully explored. Here we show that in vivo administration of anti-TNF-α Ab, using a longitudinal analysis, results in the rapid down-regulation of a spectrum of cytokines, cytokine inhibitors, and acute-phase proteins. Marked diurnal variation in the serum levels of some of these were detected. These results were consistent with the concept of a cytokine-dependent cytokine cascade, and the degree of clinical benefit noted after anti-TNF-α therapy is probably due to the reduction in many proinflammatory mediators apart from TNF-α, such as IL-6, which reached normal levels within 24 h. Serum levels of cytokine inhibitors such as soluble p75 and p55 TNFR were reduced as was IL-1 receptor antagonist. Reductions in acute-phase proteins occurred after serum IL-6 fell and included serum amyloid A, haptoglobin, and fibrinogen. The latter reduction could be of importance, as it is a risk factor for atherosclerosis, which is augmented in RA patients. ***The Journal of Immunology,*** **1999, 163: 1521–1528.**

A wide range of cytokines and other inflammatory mediators are expressed in the joint in rheumatoid arthritis (RA)[3] (1). IL-1, IL-6, GM-CSF, and the growing family of chemokines have all been proposed to play a role in disease pathogenesis (1, 2). However, the arguments that place TNF-α at the heart of the inflammatory process in RA are particularly compelling. TNF-α and its two receptors (p55 and p75 TNFR) are expressed at many sites within the synovial membrane, including the cartilage/pannus junction (3, 4). Tissue expression of these molecules is reflected in the synovial fluid, where elevated levels of TNF-α and soluble forms of the receptors (sTNFR) are seen (5–7). Using an RA synovial cell culture system in which there is spontaneous production of many cytokines (8), we showed that neutralizing TNF-α down-regulates the production of IL-1, IL-6, IL-8, and GM-CSF (9–11), and these findings led us to propose TNF-α as a therapeutic target in RA.

Direct demonstrations of the importance of TNF-α in inflammatory arthritis in vivo was first provided by animal studies. In experiments aiming to block the endogenous TNF production in arthritis, we and others showed that systemic administration of blocking Abs to TNF or of sTNFR:Fc fusion proteins after disease onset led to amelioration of joint disease in murine collagen-induced arthritis (12–14). Transgenic mice carrying a 3′-modified human TNF-α transgene showed dysregulated TNF-α expression and the development of a chronic inflammatory polyarthritis, preventable with monoclonal anti-human-TNF-α (15). Evidence that TNF-α exerted its pathogenic effect via IL-1 in this model was provided by the therapeutic benefit observed with an anti-IL-1 receptor Ab (16), reflecting the hierarchy we observed in RA cultures.

These findings led to the conclusion that TNF-α was a prime therapeutic target in RA (17, 18) and provided the rationale for clinical trials of a specific, TNF-blocking chimeric (human/mouse) mAb (cA2) in patients with RA (19–21). In the most definitive of these studies, cA2 was compared with placebo in a multicenter, randomized, double-blind trial. The outcome was unequivocal, with the induction of large and highly significant improvements in a wide range of clinical and laboratory measures of disease activity in active treatment groups (20).

There are not many opportunities to investigate the pathogenesis of human disease in vivo. The clear outcomes of cA2 therapy provides us with an opportunity to probe deeper into the role of TNF-α in the pathogenesis of RA. We have previously described the effect of cA2 administration on leukocyte migration (22), documenting a reduction in the expression of adhesion molecules and relating these changes to circulating leukocyte numbers. Here, we explore the effects of cA2 on circulating cytokines and cytokine inhibitors, and test the relationship between these mediators and their major recognized systemic effect in RA, the production of hepatic acute-phase proteins. The results confirm the central role of TNF-α in the pathogenesis of RA.

Materials and Methods

Trial procedures

The selection of patients for this study and their treatment have been described in detail elsewhere (20). In brief, 73 patients meeting the revised

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[3] Abbreviations used in this paper: RA, rheumatoid arthritis; sTNFR, soluble TNFR; IL-1ra, IL-1 receptor antagonist; CRP, C-reactive protein; SAA, serum amyloid A.

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American College of Rheumatology (ACR) criteria for the diagnosis of RA (23) were recruited from the clinics of four cooperating trial centers. All patients had active RA and evidence of erosive disease on x-rays of hands or feet. Patients taking disease modifying antirheumatic drugs were withdrawn from their therapy at least 4 wk before study entry, but were permitted to continue taking low dose oral corticosteroids or nonsteroidal antiinflammatory drugs at stable dosage.

cA2 (Remicade, Infliximab; Centocor, Malvern, PA) is a human/murine chimeric mAb of IgG1κ isotype, with specificity for recombinant and natural human TNF-α (24). At entry to the study, patients were randomized to receive a single 2 h infusion of either placebo (0.1% human serum albumin, 24 patients), low-dose cA2 (1 mg/kg, 25 patients), or high-dose cA2 (10 mg/kg, 24 patients) as an outpatient procedure. Patients were then followed using clinical and laboratory parameters for a period of 4 wk. Patients, investigators, and laboratory personnel were blinded as to the treatment administered.

Blood samples for laboratory measurements were drawn before the infusion on day 0 and at the following times after completion of the infusion: 1 and 8 h, 1 and 3 days, 1, 2, 3, and 4 wk. This resulted in the following mean collection times: 0830, 1300, 2000 (on day 0); 1200, 1100 (on days 1 and 3); 1030, 1100, 1045, 1045 (on weeks 1–4). Blood was collected into sterile tubes, allowed to clot for 30 min, and spun at room temperature for 20 min at 2500 rpm. Serum was aliquoted into plastic tubes and stored at −70°C until assayed. Plasma was prepared from EDTA blood and handled similarly.

Laboratory measurements

Laboratory measurements were made using commercially available assays where available according to the manufacturers' directions. All samples from a given patient were assayed together to reduce interassay variability.

Cytokines and inhibitors. TNF-α, IL-6, and IL-10 were measured using enzyme-amplified sensitivity immunoassays, each based on an oligoclonal detection system (Biosource, Fleurus, Belgium). The sensitivity of the assays was 10 pg/ml (TNF-α and IL-6) and 4 pg/ml (IL-10). IL-1 receptor antagonist (IL-1ra) was measured using a quantitative sandwich enzyme immunoassay (R&D Systems Europe, Abingdon, U.K.). The sensitivity of the assay was 94 pg/ml.

IL-1β is difficult to measure in serum, but we have previously investigated a number of different assays for its measurement. IL-1β was measured using a solid phase ELISA. Monoclonal anti-IL-1β 58.121.08 (Biosource) was coated onto a microtiter plate overnight at 4°C. Free binding sites were blocked using 3% BSA in PBS (0.15 M, pH 7.2). Excess blocking solution was washed away using 0.1% Tween in 0.8% saline. then, 200 μl of samples or standards were added in duplicate to the wells together with 50 μl of biotin conjugated mAb 58.121.03 (Biosource). The plates were then incubated for 2 h at room temperature. Unbound material was washed away using 0.1% Tween in 0.8% saline. Avidin-peroxidase (Boehringer Mannheim, Lewes, U.K.) was added to the wells and incubated at room temperature for 30 min. Unbound conjugate was then washed away. The reaction sites were amplified using Biotynal tyramide (DuPont, Stevenage, U.K.) at room temperature for 15 min, and then unbound material was washed away. Avidin-peroxidase (Boehringer Mannheim) was added to the wells and incubated at room temperature for 30 min. Unbound conjugate was then washed away, tetramethylbenzidine (Sigma, Poole, U.K.) was added to visualise the reaction, and the resulting color following addition of 50 μl 0.1 M sulfuric acid to each well was read at 450 nm. The OD obtained were compared with those obtained from a standard curve made from dilutions of rIL-1β (Biosource), and the concentration of IL-1β in each sample calculated. The sensitivity of this assay was found to be 0.5 pg/ml. Analysis was restricted to the placebo and 10 mg/kg cA2-treated groups to conserve on samples and reagents.

Soluble p55 and p75 TNFR were measured using an in house immunoassay, according to the method previously described (7, 25). Briefly, mAb to either p55 or p75 receptor were coated onto microtiter plates and incubated overnight at 4°C. Free binding sites were blocked with 3% BSA/PBS. Diluted standard or serum was added to each well and incubated for 2 h at room temperature. Unbound material was then washed away. Bound receptor was detected using complementary mAbs conjugated to biotin. After washing, incubation with streptavidin-peroxidase, further washing, and incubation with tetramethylbenzidine, the reaction was stopped with sulfuric acid. OD values obtained at 450 nm were compared with those obtained from a standard curve constructed using recombinant p55 and p75 TNFR. The standard curve covered a range of values from 200 pg/ml to 12.5 ng/ml. The sensitivity of each assay was 50 pg/ml. The normal values obtained from a panel of blood donors was <1000 pg/ml for p55 TNFR and <2000 pg/ml for p75 TNFR.

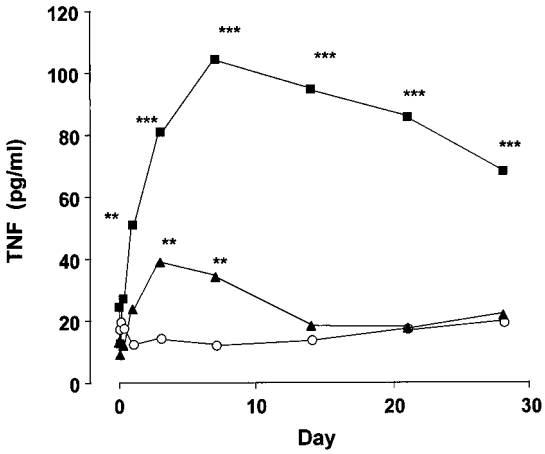

FIGURE 1. Effect of cA2 on circulating immunoreactive TNF-α. Patients were treated on day 0 with a single, 2-h infusion of either placebo (○), 1 mg/kg cA2 (▲), or 10 mg/kg cA2 (■). Each point represents the median circulating immunoreactive TNF-α level in up to 24 patients, with interquartile ranges omitted for clarity. **, $p < 0.01$; ***, $p < 0.001$ compared with placebo, by ANOVA.

Acute-phase proteins. C-reactive protein (CRP) was measured by fluorescent polarization immunoassay using the TDX system (Abbot Diagnostics, Maidenhead, U.K.). The system works by comparing the polarization value obtained for a given sample to a precalibrated standard value. Each assay was validated by the inclusion of control sera containing known quantities of CRP. Serum amyloid A (SAA) was measured by a solid phase ELISA (Biosource).

Haptoglobin and fibrinogen were measured using radial immunodiffusion (Behring, Hounslow, U.K.). EDTA plasma was placed into a well cut into a gel containing Abs to haptoglobin or to fibrinogen. After 48 h (haptoglobin) or 18 h (fibrinogen), the diameter of the resulting precipitin rings was measured and the concentration compared with a predetermined concentration table. The assays were validated by the inclusion of control sera of known concentration. These assays were confined to high-dose (10 mg/kg cA2) and placebo-treated groups to conserve samples and reagents. CRP results for all treated groups have been reported previously (20).

Statistics

Data are expressed as median, interquartile range. Diurnal variation in sTNFR, IL-1ra, and IL-6 was assessed in the placebo group using the Wilcoxon signed rank test. ANOVA on the van der Waerden normal scores was used to compare baseline values of TNF-α, IL-6, IL-1β, sTNFR, IL-1ra, CRP, and SAA as well as for comparison of changes from baseline at each posttreatment point. The model included terms for both investigational site and treatment group. Significant differences were further tested by Dunnett's comparison to the placebo group. The Mann-Whitney *U* test was used to compare the IL-10, haptoglobin, and fibrinogen data for the placebo and high-dose cA2 groups. Comparison between the percent reductions in IL-6, CRP, and SAA in the high-dose cA2 group was made using the Kruskal-Wallis test. Associations between parameters were defined using Spearman's rank correlation coefficient (ρ). No adjustment was made for multiplicity of time points or laboratory parameters. Analyses were performed on a VAX computer using SAS (SAS Software, NC) and on a PowerMacintosh computer using Minitab (Minitab, PA).

Results

Effect of cA2 on TNF-α and sTNFR

Our first aim was to examine the effect of cA2 on the systemic homeostasis of TNF-α. We could detect circulating immunoreactive TNF-α in baseline serum samples in 39 of 72 patients tested (54%). Median, interquartile range baseline circulating TNF-α levels in the placebo group were 15.5, 5–23 pg/ml ($n = 24$), for the 1 mg/kg-treated group were 5, 5–19 pg/ml ($n = 24$), and for 10 mg/kg-treated group were 12, 5–28 pg/ml ($n = 24$). The normal range is 10 pg/ml. Patients treated with placebo showed no change in circulating immunoreactive TNF-α over the 28-day

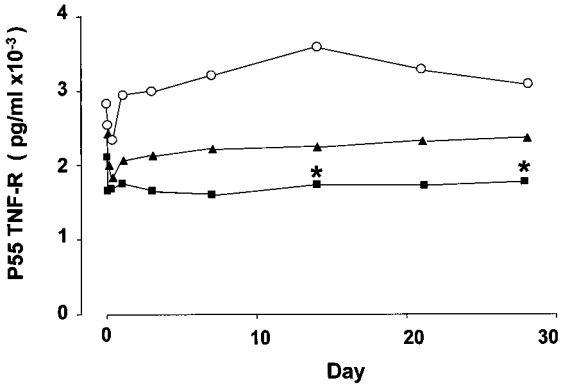

FIGURE 2. Effect of cA2 on circulating p55 sTNFR. Patients were treated on day 0 with a single, 2-h infusion of either placebo (○), 1 mg/kg cA2 (▲), or 10 mg/kg cA2 (■). Each point represents the mean circulating p55 sTNFR with ranges omitted for clarity. *, $p < 0.05$ compared with placebo by ANOVA.

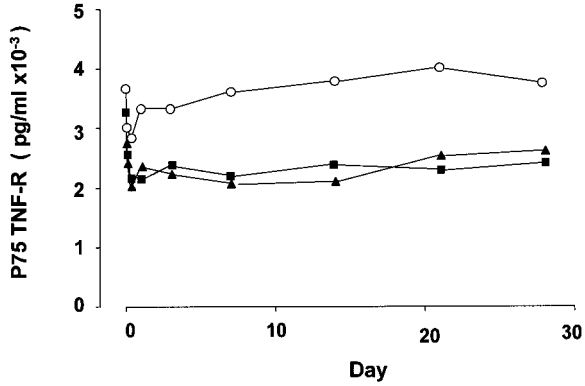

FIGURE 3. Effect of cA2 on circulating p75 sTNFR. Patients were treated on day 0 with a single, 2-h infusion of either placebo (○), 1 mg/kg cA2 (▲), or 10 mg/kg cA2 (■). Each point represents the mean circulating p75 sTNFR with ranges omitted for clarity. There were no significant differences between cA2- and placebo-treated groups at any point by ANOVA.

course of this study (Fig. 1). In contrast, patients treated with cA2 showed a rapid and highly significant increase in median circulating TNF-α, with evidence of a clear dose response relationship (Fig. 1). In patients treated with low-dose cA2, median circulating TNF-α levels peaked at day 3 and thereafter showed a gradual decline, with a return to baseline values by day 21. Peak median values in the high-dose cA2 group were seen at day 7 and showed a more gradual decline, with continuing significant elevations relative to baseline at day 28. To determine whether the circulating TNF-α was biologically active, we tested samples from several patients with high circulating immunoreactive TNF-α in the WEHI 164 TNF-α bioassay. No patient showed biologically active TNF-α either before or after treatment with cA2 (data not shown).

Normal sera contains sTNFR, both p55 and p75, and these are elevated in RA patients. It was of interest to evaluate the effect of therapy with anti-TNF-α on levels of these TNF inhibitors. p55 sTNFR was detectable in baseline serum samples from all 73 patients, with 67 (92%) showing values above the normal range (<1000 pg/ml). Median, interquartile range circulating p55 sTNFR levels at baseline for the placebo, low-dose, and high-dose cA2 groups were 2050, 1360–3683 pg/ml ($n = 24$), 2050, 1710–3130 pg/ml ($n = 25$), and 1910, 1345–2670 pg/ml ($n = 24$), respectively. The changes in circulating p55 sTNFR are shown in Fig. 2. p55 levels fell in the placebo group during the course of day 0, with trough values reported at 1300 h ($p = 0.038$ compared with baseline). By 1200 h on day 1, p55 levels in the placebo group had returned to baseline values. Median p55 levels in the two cA2-treated groups showed a similar rapid decline over the morning of day 0 but in contrast to the placebo group, remained below baseline values on day 1. Overall, patients treated with placebo showed no significant change over this period, while patients treated with low- or high-dose cA2 showed modest reductions in circulating p55, which were maximal by day 3 and showed significance ($p < 0.05$) compared with placebo at day 14 and 28. However, median values remained above the upper limit of normal in all treatment groups.

p75 sTNFR was also detectable in all 73 patients at baseline, with 54 (74%) showing baseline values above the normal range (<2000 pg/ml). Median, interquartile range circulating p75 sTNFR at baseline for the placebo, low-, and high-dose cA2 groups were 3313, 1988–5075 pg/ml ($n = 24$), 2560, 1770–3315 pg/ml ($n = 25$), and 2812, 2049–3778 pg/ml ($n = 24$), respectively. Although median values were higher for the placebo group than for the two cA2 groups, this difference was not statistically significant. Changes in p75 sTNFR during day 0 and day 1 were similar to those seen for p55, with reduction in all three treatment groups at 1300 h and 2000 h on day 0, recovery to near baseline values in the placebo group by 1200 h on day 1, but continuing suppression of values for the two cA2 groups at this time. The median p75 sTNFR values are similar over the 28 days of the study (Fig. 3) in the placebo group, but show a reduction in both cA2 treatment groups, although the changes failed to reach statistical significance compared with placebo.

Effect of cA2 on circulating IL-1 and IL-1ra

While IL-1 is clearly produced in the joints in RA, there is not much present in the serum (26). To establish the normal range for serum IL-1β using our assay, we tested serum samples obtained from 36 normal individuals. IL-1β was detectable (>0.5 pg/ml) in 11 of 36 samples (31%), with values ranging from 1 to 128 pg/ml. The median value and interquartile range were <0.5, <0.5–48.0 pg/ml.

Using the same assay, we measured IL-1β in serum samples from placebo-treated patients ($n = 24$) and patients treated with high-dose cA2 ($n = 22$) only. IL-1β was detectable in 33 of 46 patients at baseline (72%) with median, interquartile range values of 5.8, <0.5–23.1 pg/ml and 8.4, <0.5–28.1 pg/ml in the placebo and high-dose cA2 groups, respectively. Changes in serum IL-1β levels in the two RA treatment groups during the course of the study are shown in Fig. 4. The median values throughout the study remained well within the range established earlier for normal individuals. However, there was no statistically significant difference between the two groups, and no clear cut reduction over the 28 days analyzed.

Circulating IL-1ra was detectable in all 68 pretreatment sera tested, with most showing values above the manufacturers quoted normal range (<375 pg/ml). Median, interquartile range pretreatment values were not significantly higher in the placebo group (613, 485–855 pg/ml; $n = 24$) than in the two active treatment groups (525, 235–760 pg/ml, $n = 23$; 470, 275–620 pg/ml, $n = 21$; low- and high-dose cA2, respectively).

The changes in circulating IL-1ra occurring during day 0 and day 1 are shown in Fig. 5. IL-1ra levels fell in the placebo group during the course of day 0, with trough values recorded at 2000 h, ($p = 0.026$ compared with pretreatment). By 1200 h on day 1, IL-1ra levels had recovered in the placebo group, with median values similar to those observed pretreatment. The early changes

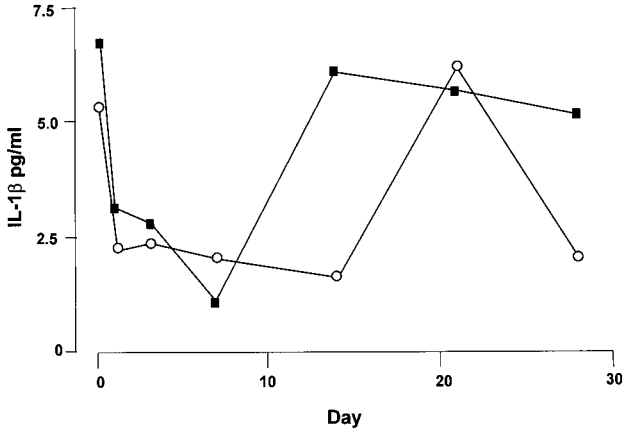

FIGURE 4. Effect of cA2 on circulating IL-1β. Patients were treated on day 0 with a single, 2-h infusion of either placebo (○) or 10 mg/kg cA2 (■). Each point represents the median circulating immunoreactive IL-1β, with interquartile ranges omitted for clarity. There were no significant differences between cA2- and placebo-treated groups at any point by ANOVA.

seen in the two cA2 groups were similar in kinetics, but greater in magnitude than in the placebo group and failed to show recovery on day 1. The reduction from pretreatment values in the high-dose cA2 group was statistically significant compared with the reduction in placebo patients at this time ($p < 0.01$).

Changes in circulating IL-1ra in the same treatment groups over a 4-wk period are shown in Fig. 5. Overall, patients treated with placebo showed no significant change over this period, while patients treated with low- or high-dose cA2 showed rapid and substantial reductions in circulating IL-1ra, which were maximal by day 3 and highly significant compared with placebo ($p < 0.001$). The reduction in IL-1ra remained significant until day 14 in both groups. Although the maximal fall seen with the treatment dose groups was similar, patients treated with low-dose cA2 showed a more rapid loss of effect, with a return to pretreatment values by week 4.

The changes in median IL-1ra values were reflected in the individual patient responses for IL-1ra. Of the 23 low-dose cA2 patients tested, 16 had elevated circulating IL-1ra levels before treatment, and, following treatment, 7 of these achieved a serum value the normal range. Similarly, 14 of the 21 high-dose cA2 patients tested had elevated pretreatment IL-1ra levels, and, following treatment, 10 achieved normal values.

Effect of cA2 on IL-6

IL-6 was detectable in all but 4 of the 72 pretreatment sera tested. The median, interquartile range pretreatment circulating IL-6 levels for the three treatment groups were 125, 56–209 pg/ml, $n = 24$; 130, 57–225 pg/ml, $n = 24$; 114, 78–188 pg/ml, $n = 24$ (normal range, <10 pg/ml) (placebo, low-, and high-dose cA2, respectively, $p < 0.05$ for each group v/s normal).

The changes in circulating IL-6 following treatment are shown in Fig. 6. IL-6 levels showed significant reductions in the placebo group at the 1300 and 2000 h time points on day 0 ($p < 0.001$, $p = 0.002$, respectively), with partial recovery by day 1 (Fig. 6). Patients treated with cA2 showed even more marked reductions in circulating IL-6 at 1300 h on day 0 and continuing decline thereafter, reaching significance compared with placebo by day 1 ($p < 0.01$, $p < 0.001$, low- and high-dose cA2, respectively). In Fig. 6, changes in serum IL-6 over the longer term are displayed. The highly significant falls in serum IL-6 seen at day 1 were maintained for the duration of the study in patients receiving high-dose cA2, but there was a partial loss of effect in patients treated with low-dose cA2 by week 4.

The changes in median IL-6 values were reflected in the individual patient responses for IL-6. Of the 24 low-dose cA2 patients tested, 22 had elevated IL-6 values pretreatment and 14 of the 22 (64%) had values within normal limits by day 1. Similarly, 23 of 24 high-dose cA2 patients had elevated circulating IL-6 pretreatment, of whom 16 (70%) had reverted to normal values by day 1.

Effect of cA2 on IL-10

While IL-10 is abundant in synovium, the levels reported in serum are lower (27). IL-10 was measured in patients receiving placebo, ($n = 18$) and in those receiving high-dose cA2 ($n = 18$) only. Of these, nine patients (24%) had detectable IL-10 at baseline (>4 pg/ml). Median, interquartile range circulating IL-10 levels at baseline were 2, 2–10.5 pg/ml and 2, 2–3.5 pg/ml in the placebo and high-dose cA2 groups, respectively (normal range, <8.8 pg/ml). There was no significant change in values in either treatment group during the course of the study (Table I).

Effect of cA2 on acute-phase proteins

The changes in circulating CRP values in this study have been reported previously (20). In brief, patients treated with placebo

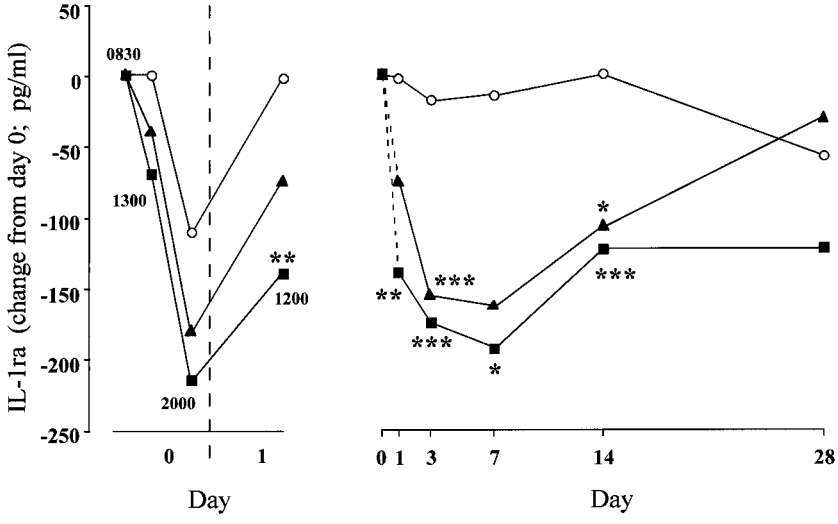

FIGURE 5. Effect of cA2 on circulating IL-1ra. Patients were treated on day 0 with a single, 2-h infusion of either placebo (○), 1 mg/kg cA2 (▲), or 10 mg/kg cA2 (■). A detailed time/response profile on day 0 and 1 is shown in *A*, with the mean sampling times indicated on the figure. Changes in circulating IL-1ra in the same three patient groups over the longer term are shown in *B*. Each point represents the median circulating immunoreactive TNF-α level in up to 24 patients, with interquartile ranges omitted for clarity. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with placebo, by ANOVA.

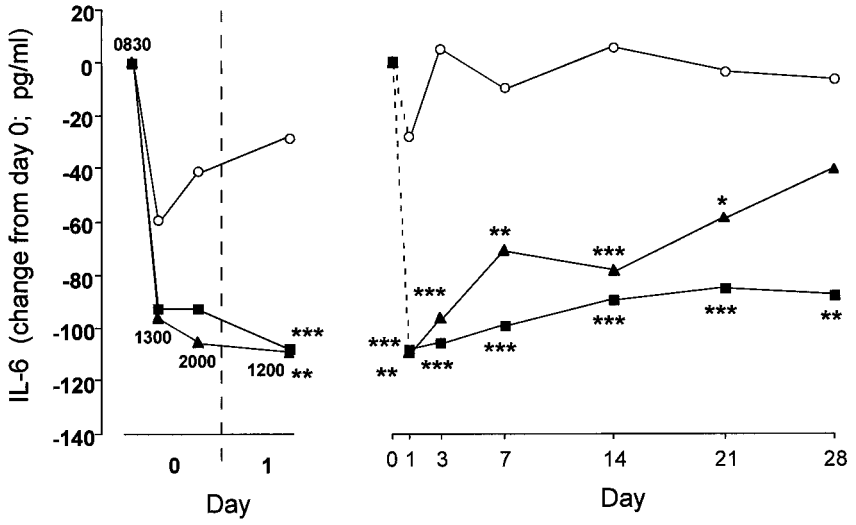

FIGURE 6. Effect of cA2 on circulating IL-6. Patients were treated on day 0 with a single, 2-h infusion of either placebo (○), 1 mg/kg cA2 (▲), or 10 mg/kg cA2 (■). A detailed time/response profile on day 0 and 1 is shown on the *left*, with the mean sampling times indicated on the figure. Changes in circulating IL-6 in the same three patient groups over the longer term are shown on the *right*. Each point represents the median circulating IL-6 level in up to 24 patients, with interquartile ranges omitted for clarity. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with placebo, by ANOVA.

showed no significant change in CRP values, while those treated with high-dose cA2 showed a large and highly significant reduction, evident as early as day 1 posttreatment and reaching maximal extent by day 7 (Table II).

Pretreatment circulating SAA were similar in both placebo and high-dose treatment groups (335, 62–750, mg/L, $n = 24$; 378, 180–935 mg/L, $n = 24$; placebo and high-dose cA2, respectively; normal range, <10 mg/L). No significant changes in SAA levels were seen following treatment in the placebo group (Table I), while those patients treated with high-dose cA2 showed a large and highly significant reduction.

Pretreatment haptoglobin and fibrinogen levels were similar in the placebo and high-dose cA2 treatment groups (Table II). Placebo-treated patients showed no significant change in either measure, while the high-dose cA2 group showed a significant reduction in both measures by week 4 ($p < 0.001$, $p < 0.002$, haptoglobin and fibrinogen, respectively).

Association between circulating IL-6 and acute-phase proteins

Because IL-6 is the principal regulator of hepatic acute-phase protein synthesis in vitro (28, 29) and in vivo in IL-6 gene-targeted mice (30), we tested the association between IL-6, CRP, and SAA in our patients. A comparison of the kinetics of change in each of these mediators following treatment with high-dose cA2 showed falls in IL-6 preceding those of CRP or SAA. By day 1, median circulating IL-6 values had fallen compared with pretreatment values by 95% and exceed the reductions in CRP (20%) and SAA (5%) ($p < 0.001$). Although the median values for the two acute-phase proteins had fallen further by day 3, thereby narrowing the gap with IL-6, the reduction in IL-6 at this time was still significantly greater than for the acute-phase proteins ($p < 0.001$). A scatter graph comparing pretreatment IL-6 and CRP levels in all 73 patients is shown in Fig. 7*A*, indicating a moderate association between these variables ($\rho = 0.55$, $p < 0.002$). A similar association was found when comparing the reduction in circulating IL-6 by day 3 with the reduction in CRP over the same time period (Fig. 7*B*; $\rho = 0.59$, $p < 0.002$). Less impressive, but still statistically significant, associations were seen between circulating IL-6 and SAA (pretreatment comparison: $\rho = 0.44$, $p < 0.002$; reduction by day 3 comparison: $\rho = 0.48$, $p < 0.002$). The strongest associations observed were between CRP and SAA (pretreatment comparison: $\rho = 0.73$, $p < 0.002$; reduction by day 3 comparison: $\rho = 0.76$, $p < 0.002$).

Table I. *IL-10*[a]

Day	Placebo	10 mg/kg cA2	*p* Value
0	2,2–10	2,2–8	NS
7	2,2–15	2,2–3	NS
14	2,2–9	2,2–3	NS
28	2,2–8	2,2–3	NS

[a] The data are expressed as median, interquartile range. There were no significant differences between the two treatment groups for any measure prior to or following treatment. Values of *p* assess significance of the change from pretreatment values in the cA2 group compared with change in the placebo group by ANOVA or Mann-Whitney *U* test.

Discussion

The analysis of cytokine expression and regulation in rheumatoid arthritis has led to the definition of new therapeutic targets for this

Table II. *Acute-phase proteins*[a]

Day	Placebo	10 mg/kg cA2	*p* Value
CRP (mg/L)	$n = 24$	$n = 24$	
0	56, 33–70	65, 28–94	
1	52, 27–63	55, 22–71	0.05
3	47, 31–74	22, 13–31	0.001
7	56, 31–72	18, 13–35	0.001
14	49, 29–72	19, 10–44	0.01
28	60, 29–71	24, 13–50	0.001
SAA (mg/ml)	$n = 24$	$n = 24$	
0	335, 62–750	378, 180–935	
1	420, 127–755	380, 121–908	NS
3	441, 115–890	50, 19–163	0.01
7	378, 136–810	62, 26–206	0.01
14	493, 228–853	56, 26–272	0.01
28	720, 176–887	69, 22–345	0.01
Haptoglobin (g/L)	$n = 20$	$n = 15$	
0	3.4, 3.0–4.3	3.3, 2.7–3.7	
28	3.4, 3.0–4.3	2.5, 1.5–3.2	0.001
Fibrinogen (g/L)	$n = 20$	$n = 14$	
0	3.8, 3.4–5.0	4.1, 3.2–4.7	
28	4.0, 3.3–5.4	2.8, 2.2–3.1	0.002

[a] The data are expressed as median, interquartile range. There were no significant differences between the two treatment groups for any measure prior to treatment. Values of *p* assess significance of the change from pretreatment values in the cA2 group compared with change in the placebo group by ANOVA (CRP, SAA) or Mann-Whitney *U* test (haptoglobin, fibrinogen). Normal ranges: CRP, <10 mg/L; SAA, <10 mg/L; haptoglobin, 0.7 –3.8 g/L; fibrinogen, 1.8 –3.5 g/L.

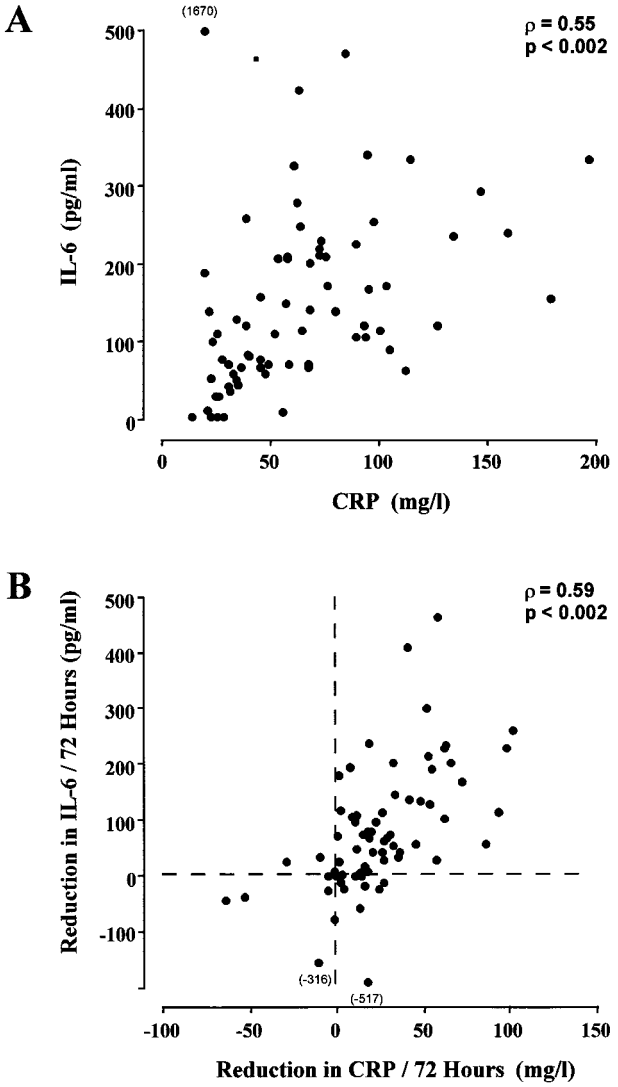

FIGURE 7. The relationship between circulating IL-6 and CRP. *A*, The relationship between circulating IL-6 and CRP in all 73 patients pretreatment (Spearman's rank correlation coefficient (ρ) = 0.55, $p < 0.002$) is shown. *B*, The relationship between the reduction in circulating IL-6 by day 3 and the reduction in CRP over the same period in all 73 patients (ρ) = 0.59, $p < 0.002$) is shown. Each point represents an individual patient.

disease (1, 31). Proinflammatory cytokines have been the targets of biological therapy in RA in recent years, including IL-1, IL-6, and TNF-α. It is blockade of the latter that has had the most success, with reproducible clinical data using mAbs (cA2, Remicade, Centocor; CDP571, Celltech, Berkshire, U.K.) and fusion proteins (p75Fc, Enbrel, Immunex, Seattle, WA; p55Fc, Lenercept, Roche, Gipf-Oberfrick, Switzerland).

The precise molecular targeting of biological agents that remain outside the cell and do not have multiple targets makes it possible to analyze and interpret the changes occurring after the therapy to shed new insights into the pathogenesis of disease. The large and reproducible effects of cA2 provided an opportunity to investigate the consequences of neutralizing TNF-α and other cytokines and cytokine inhibitors. In particular, we wanted to know if the interactions we found using in vitro joint cell cultures also applied in vivo. Although the most important cytokine actions in RA are likely to be within the joint, the relative inaccessibility of synovial tissue during clinical trials makes direct study of this compartment difficult and the assays that can be used in synovium such as immunohistochemistry or PCR are not very quantitative. Here, we report an analysis of the effects of cA2 on cytokines within the circulation and relate changes in their levels to a key cytokine-dependant biological effect, the production of acute-phase proteins (31).

Our first aim in undertaking this study was to analyze changes in systemic TNF homeostasis following infusion of cA2. Approximately half of the patients had detectable circulating TNF-α at baseline, but levels were generally low. The changes that occurred following infusion of cA2 were remarkable, with a rapid and dose-dependent increase in immunoreactive, but not biologically active, TNF-α, evident as early as 8–24 h and peaking by day 7. While the mechanism of this increase in immunoreactive TNF-α is still under investigation, preliminary evidence suggest that the TNF-α is present in the form of a high m.w. complex, presumably with cA2 (our unpublished observations). Similar increases in circulating immunoreactive IL-6 were noted following treatment with a mAb to IL-6 in patients with RA (32) and plasma cell leukaemia (33). Therefore, the rapid, but transiently delayed (not present at 4 h) rise in immunoreactive TNF-α in our patients is likely to represent trapping of the TNF-α overproduced in the disease.

Changes in sTNFR following cA2 administration were in the opposite direction of those seen for TNF-α itself. Both p55 and p75 sTNFR were rapidly diminished following infusion of the mAb, reaching statistical significance compared with placebo for p55 sTNFR at several time points. Interestingly, although levels of sTNFR were markedly reduced by 1300 h on day 0 in both low- and high-dose cA2 groups, a similar reduction was observed for the placebo group throughout day 0. These findings strongly suggest a diurnal variation for sTNFR, although the changes might also have resulted from some unknown factor inherent in the infusion procedure with either cA2 or placebo, and further studies are warranted. The observed reductions in sTNFR following administration of cA2 occurred in parallel with improvement in disease activity in this patient group (20) and support our earlier findings showing a relationship between levels of sTNFR and disease activity in RA (7).

The investigation of changes in IL-1 and its competitive antagonist, IL-1ra, was a second important aim of this study. IL-1 shares many biological functions with TNF-α and is particularly potent in the induction of damage to cartilage and bone, which led some to consider it the prime therapeutic target in RA (34). On the basis of in vitro experiments (9), we predicted that effective TNF-α neutralization in vivo would lead to a down-regulation of IL-1 production. However, although we saw a downward trend in circulating IL-1β in patients treated with high-dose cA2 at 7 days, the changes were not significant compared with placebo and took place entirely within the previously established normal range. IL-1 is clearly expressed within the synovial membrane (35–37) and in blood mononuclear cells in RA (38, 39), but has been difficult to detect in the circulation (26) which led us to investigate a number of assays. Our data is in accord with these findings and suggest that the great majority of synovial IL-1 production is consumed or degraded locally or is rapidly complexed with natural inhibitors such as soluble IL-1R either within or following release from the joint. A prior study on a subset of the same patients has been reported by Lorenz et al. (40), who found a reduction in serum IL-1 levels at days 1 and 7. We do not know why there is a discrepancy; it may be due to the assay used (i.e., whether it is receptive to soluble IL-1R) or the patients. Further work may clarify this.

The biological effect of IL-1 depends upon the ratio of this cytokine to its competitive antagonist, IL-1ra (41). Analysis of IL-1ra

in serum samples in our study showed elevated pretreatment levels in the majority of patients. Treatment with cA2 lead to substantial falls in circulating IL-1ra, reaching statistical significance compared with placebo by day 1. The data support the findings of Van der Poll and colleagues, who showed reduced circulating IL-1ra after treatment of experimental endotoxemia with mAb to TNF-α (42). The rapidity and timing of this change suggest that TNF-α has an important role in the regulation of IL-1ra release.

Analysis of changes in IL-1ra at the early time points was complicated by the significant reduction in circulating IL-1ra seen in the placebo group on day 0. The pattern was similar to that seen for sTNFR and suggests a diurnal rhythm in levels of cytokine antagonists in RA, although as discussed previously, other factors inherent in the infusion process cannot be ruled out. Although the differences between the placebo and cA2 groups were not statistically significant at the early time points on day 0, the greater falls in cA2-treated patients and the trend toward a dose-response relationship (Fig. 5) suggest that regulation of IL-1ra expression by cA2 is very rapid. It has been reported that IL-1ra is made by hepatocytes and by cells within the joint and behaves as an acute-phase protein.

The detection of elevated circulating IL-6 in our patients is consistent with previous reports that showed the presence of IL-6 in the majority of RA sera, although at lower concentrations than found in matched synovial fluid samples (42–46). The reductions in circulating IL-6 in placebo-treated patients during the course of days 0 and 1 are consistent with the recognized diurnal variation in this cytokine in patients with RA (45). The reductions in circulating IL-6 in cA2-treated patients were even more marked than in the placebo group, reaching significance from day 1. These findings support our earlier, preliminary report of reductions in circulating IL-6 in patients treated in the open label trial of cA2 (19) and concur with the results of an open trial of cA2 in active, refractory Crohn's disease (47). The data provide in vivo confirmation of earlier in vitro findings that TNF-α is regulatory for IL-6 production in RA synovial tissue (11). The difference in kinetics, more rapid in vivo, is likely to be due to clearance mechanisms including excretion in vivo.

In view of ample in vitro and animal data linking IL-6 with acute-phase protein synthesis in inflammation (28, 29), it was of interest to relate IL-6 levels in our patients to the cA2-induced falls in serum acute-phase proteins (19–21). Although IL-6, CRP, and SAA all fell markedly by day 3 and showed significant correlations with each other, the rate of fall for IL-6 was clearly much more rapid than for CRP or SAA. This temporal relationship is consistent with regulation of CRP and SAA production by IL-6, but interpretation of the data is complicated by marked differences in the circulating half times of these molecules (48, 49). An association between circulating IL-6 levels and CRP in patients with inflammatory arthritis has been noted previously (45).

The impressive falls in circulating levels of the rapid response elements, CRP and SAA, following treatment with cA2 were accompanied by significant reductions in the slower response proteins, haptoglobin and fibrinogen. Although these proteins are normally measured as markers of disease activity, rather than as pathophysiological agents in their own right, acute-phase proteins may directly contribute to disease outcomes in RA. Prolonged, high-level elevation in circulating SAA is associated with the development of secondary amyloidosis, a cause of renal failure and premature death in a small proportion of RA patients (50). It has also been suggested that persistently elevated SAA may be a risk factor for cardiovascular disease (51). Potentially more important is the strong association between elevation of plasma fibrinogen levels and the development of vascular disease (52). Although other factors, including high platelet counts (19, 20), undoubtedly also contribute to the excess cardiovascular and cerebrovascular mortality seen in RA, persistently elevated fibrinogen levels may be major contributors to these adverse disease outcomes. Our data clearly show that TNF-α neutralization in the short term leads to normalization of SAA and fibrinogen levels in many patients. Whether effective long-term TNF neutralization will maintain control of these proteins and as a result lead to a reduction in the development of amyloidosis and vascular disease remains to be seen. The normalization of thrombocytosis, which we have previously reported following treatment of RA patients with cA2 (19, 20), also has the potential to lower cardiovascular risk, because platelets play an important role in both early and late events in the development of the atherosclerotic lesion (53).

The findings presented here support the notion of a cytokine cascade in RA patients in vivo, with regulation by TNF-α of IL-6 and of the important antiinflammatory molecules, IL-1ra, soluble p55 TNFR, and soluble p75 TNFR. The establishment of a relationship between TNF and the regulation of the acute-phase response adds biological significance to these findings, provides a model that may explain other beneficial clinical effects of TNF neutralization in this disease, and helps provide a rationale for the use of CRP in monitoring the activity of RA.

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