

CAVELIER

ABOGADOS

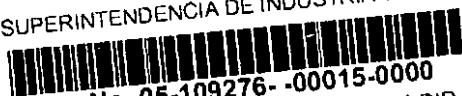
www.cavelier.com
cavelier@cavelier.com

EDIFICIO SISKI
CARRERA 4 No. 72 - 35
BOGOTÁ 8, COLOMBIA

Teléfono: (57-1) 347 3611
Telefax: (57-1) 211 8650
(57-1) 700 4814
Fax in U.S.A.: (1-305) 675 7743

Señora Directora
DIRECCION DE NUEVAS CREACIONES
Superintendencia de Industria y Comercio
E. S. D.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 05-109276-00015-0000
Fecha: 2010-12-23 16:55:12 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYII Eve: 379 FASENACIONALII
Act. 444 RESPUESTAREQUE Folios: 21

Trámite : 011
Evento : 379
Actuación : 444

Referencia : Expediente No. 05.109.276
Asunto : Solicitud de patente para **ANTAGONISTAS DE INTERLEUKIN-6 Y COMPOSICIONES FARMACÉUTICAS QUE LOS CONTIENEN**
Solicitante : **CHUGAI SEIYAKU KABUSHIKI KAISHA**
Fecha de Solicitud : 26 de octubre de 2005

1. Yo, Luz Clemencia DE PÁEZ, en mi condición de apoderada de la sociedad solicitante de la patente de la referencia, me refiero al oficio No. **10483** notificado por fijación en lista del 20 de agosto de 2010.

2. Mediante memorial radicado el día 11 de noviembre de 2010 solicité plazo de treinta (30) días para atender el oficio No. **10483**.

3. Mediante oficio No. **10483** se puso en conocimiento del solicitante el informe que contiene las observaciones hechas por su Despacho sobre la presente solicitud de patente. Las observaciones realizadas en el oficio son las siguientes:

3.1 Descripción

Afirma el Examinador que no se encuentra soporte para todas las reivindicaciones y que en el capítulo descriptivo solo se encuentra soportada la administración simultanea del anticuerpo anti-IL-6R y de un inmunosupresor tal como metotrexato mediante combinación terapéutica y por lo tanto, no se encuentra evidencia acerca de la composición que contenga ambos fármacos.

3.2 Reivindicaciones

El Examinador afirma que "la reivindicación 1 referente a las composiciones farmacéuticas de un anticuerpo anti-il-6R y metotrexato, no se encuentran sustentadas en la descripción". De igual manera afirma que "la reivindicación 2 referente a las composiciones farmacéuticas de un anticuerpo anti-il-6R, el cual es un anticuerpo quimérico, humanizado o un anticuerpo de tipo humano, no se encuentran sustentadas en la descripción".

3.3 Análisis de patentabilidad

3.3.1 Nivel Inventivo

Para este requisito se considera que una persona versada en la materia enfrentada al problema resuelto por la presente invención, incluiría metotrexato de acuerdo con las enseñanzas de D3, en las composiciones reveladas en D4, logrando de esta manera las composiciones farmacéuticas de la presente invención. Por lo anterior, afirma el Examinador que la presente invención no posee nivel inventivo frente al estado de la técnica.

4. Respuesta a las objeciones

De este modo y con el objeto de atender las objeciones arriba mencionadas, se desean poner de presente las siguientes aclaraciones y argumentos:

4.1 Argumentos a favor de la objeción de falta de soporte de la materia reclamada

Con respecto a las objeciones hechas por el Examinador con respecto a la falta de soporte de la materia reclamada en la descripción deseamos poner de presente los siguientes argumentos:

Inicialmente, deseamos aclarar que las modalidades de uso combinado de metotrexato y un anticuerpo son conocidas, así como las tabletas de administración oral, otras formulaciones tal como gotas para los ojos (como por ejemplo las vendidas por Pfizer),

son conocidas. Es por esto que la descripción original de la solicitud internacional WO2004/096273 en la página 5, ultimo párrafo y en la pagina 8, en el tercer párrafo, describe que los medicamentos de la composición de la presente invención pueden ser administrados en diferentes maneras. Así, debido a que tanto el metotrexato como el anticuerpo de la presente invención pueden ser soluciones, los mismos pueden estar contenidos en una misma composición. En consecuencia, una composición que contiene tanto metotrexato como el anticuerpo de la presente invención se encuentra debidamente soportado en el capítulo descriptivo de la solicitud, ya que una persona versada en la materia encontraría la información suficiente para la realización de la misma.

Así las cosas, consideramos que las enseñanzas de la descripción de la presente solicitud son suficientes para que una persona versada en la materia desarrolle la composición reivindicada.

4.2 Argumentos a favor del nivel inventivo de la solicitud

En concordancia con el Artículo 18 de la Decisión 486, una invención se considerará inventiva si la misma no hubiese resultado obvia a partir del arte previo, para una persona del oficio normalmente versada en la materia. Esto a su vez implica que el Examinador a cargo de esta evaluación debe colocarse en el momento exacto en el que el inventor buscaba resolver el problema técnico contemplado en la solicitud y para el cual poseía la literatura y el conocimiento disponibles en dicho momento.

Siguiendo entonces los lineamientos de la Jurisprudencia aplicable al análisis del nivel inventivo de una solicitud de patente, se considera que una anterioridad o una combinación de dos anterioridades puede afectar dicho requerimiento de patentabilidad, si una persona medianamente versada en la materia encuentra enseñanzas o sugerencias claras que le indiquen cómo llegar a la invención reivindicada.

Ahora bien, con respecto al análisis realizado por el Señor Examinador, deseamos poner de presente lo siguiente:

Inicialmente, deseamos poner de presente que previo a la fecha de la prioridad de la presente solicitud era bien sabido que las citocinas como la IL-6 presentaba comportamientos complejos y se conocían por ejemplo, los siguientes:

- Una citosina mostraba varias actividades biológicas (Pleiotropía)
- Una pluralidad de citocinas mostraba la misma actividad en la misma célula (Redundancia);
- Una pluralidad de citocinas se involucraban en la misma línea de célula, dependiendo del proceso de diferenciación y del crecimiento.

Así, mediante el conocimiento previo de la fecha de prioridad de la presente solicitud, acerca de la existencia de los comportamientos complejos de las citocinas, se había reportado acerca de casos en donde IL-6 se expresaba de manera negativa en la supresión de la inflamación.

Por ejemplo, en el documento anexo a este memorial (Intratracheal Injection of Endotoxin and Cytokines, Interleukin-6 and Transforming Growth Factor Beta Inhibit Acute Inflammation, American Journal of Pathology, 138(5), 1991, 1097-1101) se describe que la interleuquina-6 muestra también ser endógenamente regulada por incremento dentro del pulmón posterior al desafío intratraqueal con endotoxina, lo cual proporciona evidencia de que IL-6 puede representar un mecanismo endógeno con retroalimentación negativa para inhibir la inflamación aguda iniciada por endotoxina y mediada por citocinas. Adicionalmente, se afirma en dicho documento que "el mecanismo de la acción antiinflamatoria de IL-6 y TGF β puede relacionarse con la habilidad de dichas citocinas para inhibir TNF α (y como se muestra en los datos presentes) y la producción de IL-1, mediante macrófagos. IL-6 derivada de un huésped es localmente regulada por incremento posterior al desafío con LPS y puede actuar como mecanismo endógeno con retroalimentación negativa para inhibir la inflamación aguda iniciada por LPS y mediada por TNF" (Página 1100, columna derecha).

Las anteriores afirmaciones son muestra de que IL-6 se expresa altamente en una parte inflamatoria en el cuerpo y que la IL-6 altamente expresada actúa de manera negativa, lo que resulta en una inflamación mediada por IL-6 o en represamiento de TNF.

Así, una persona versada en la materia puede considerar la posibilidad de que IL-6 se expresa altamente en las acciones de inhibición de IL-6 y TNF, lo que resulta en la inhibición de la inflamación.

Adicionalmente, en el documento anexo a este memorial (COMBINATION OF METHOTREXATE AND PREDNIZONE DECREASES CIRCULATING CONCENTRATIONS OF INTERLEUKIN 1B AND INTERLEUKIN 6 IN PATIENTS WITH RHEUMATOID ARTHRITIS, POOR CORRELATION OF CITOKINE SUPPRESSION WITH CLINICAL IMPROVEMENT, International Journal of Immunopathology and Pharmacology, 12(1), 13-21 (1999)) se concluye en el resumen que "el monitoreo de concentraciones de plasma de dichas citocinas no puede predecir el efecto del tratamiento", y en la pagina 20, en la columna izquierda párrafo 3, se concluye que "la supresión de dichos parámetros inducidas por el tratamiento no se relaciona la mejora del estado clínico del paciente y no puede haber sido útil para la predicción de la eficiencia del tratamiento".

En consecuencia, según lo afirmado en dicho documento, la inhibición de una citosina y el efecto terapéutico no se relacionan.

De lo anterior, cabe aclarar que con respecto a las enfermedades relacionadas con citocinas, no existe una correspondencia entre el hecho de que una citocina se relaciona a una causa de una enfermedad y que la inhibición de una citocina resulta en la inhibición de la enfermedad. Adicionalmente, puede existir un caso en donde la inhibición de la citocina empeore la enfermedad.

En conclusión, considerando el hecho de que en varias publicaciones, como las anexadas a este memorial, se conoce que no existe relación entre el hecho de que IL-6 es altamente expresado y en que el efecto terapéutico se obtiene mediante la inhibición de

IL-6, una persona versada en la materia, tal como lo afirma el Examinador, no habría encontrado motivación alguna en las anterioridades citadas para usar un inhibidor de IL-6 para un tratamiento de una enfermedad, motivada por el hecho de que IL-6 es altamente expresada en dicha enfermedad, debido a que este hecho no puede ser base para determinar si un inhibidor de IL-6 puede ser usado para el tratamiento de una enfermedad o no.

Así las cosas, aunque no existiría una posibilidad en cuanto a que el metotrexato disminuyera la concentración de IL-6, sería imposible predecir que el metotrexato al ser usado en combinación con un anticuerpo antagonista del receptor IL-6, se obtenga un efecto sinérgico.

De esta manera, el hecho de que D3 mencione el metotrexato en AR frente a las interleuquinas IL-6 puede ser considerada una razón válida para no reconocer el nivel inventivo de la presente solicitud, toda vez que el análisis ex post facto de una solicitud de patente se encuentra proscrito ya que el Examinador debe situarse en la fecha de la prioridad reivindicada y sin tener en cuenta la solución aportada por la invención en cuestión, el análisis de nivel inventivo en el presente caso fue efectuado erróneamente y por ende no resulta válido de acuerdo con los parámetros del Manual Andino de Patentes.

De esta manera, una persona versada en la materia solo consideraría D3 si ya conociera la presente invención, lo que indica que la combinación de las enseñanzas de D4 y D3 resultaría obvia solamente a posteriori.

Por consiguiente, teniendo en cuenta los argumentos presentados, se concluye que las enseñanzas de las anterioridades citadas por su Despacho no llevan a un técnico versado en la materia a obtener la presente invención ya que no revelan o enseñan las características técnicas esenciales relacionadas con la misma, tal como acá es reivindicada. Lo anterior si se tiene en cuenta que para el estudio de nivel inventivo el Examinador no puede adoptar la posición de un inventor y que además no puede partir de la solución dada sino simplemente de lo que expresamente revelen el o los documentos más cercanos del estado del arte.

Puesto que se han atendido a cabalidad las observaciones hechas por su Despacho sobre la presente solicitud de patente, respetuosamente solicito se continúe con su tramitación. Sin embargo, en el evento que el Despacho considere que una modificación adicional es necesaria, respetuosamente le solicito hacer uso de la facultad que le otorga el artículo 45 inciso 2 de la Decisión 486 de la Comisión de la Comunidad Andina a efecto de requerir nuevamente de nuestra parte cualquier complementación o aclaración.

4. ANEXOS

- **ANEXO I:** Intratracheal Injection of Endotoxin and Cytokines, Interleukin-6 and Transforming Growth Factor Beta Inhibit Acute Inflammation, American Journal of Pathology, 138(5), 1991, 1097-1101
- **ANEXO II:** COMBINATION OF METHOTREXATE AND PREDNIZONE DECREASES CIRCULATING CONCENTRATIONS OF INTERLEUKIN 1B AND INTERLEUKIN 6 IN PATIENTS WITH RHEUMATOID ARTHRITIS, POOR CORRELATION OF CITOKINE SUPPRESSION WITH CLINICAL IMPROVEMENT, International Journal of Immunopathology and Pharmacology, 12(1), 13-21 (1999))

Señora Directora,


LUZ CLEMENCIA DE PÁEZ

C.C. 35.456.344 de Usaquén

T.P.A. No. 23.555

Rapid Communication

Intratracheal Injection of Endotoxin and Cytokines

II. Interleukin-6 and Transforming Growth Factor Beta Inhibit Acute Inflammation

Thomas R. Ulich,* Songmei Yin,* Kaizhi Guo,*
Eunhee S. Yi,* Daniel Remick,† and
Juan del Castillo*

From the Departments of Pathology, the University of California at Irvine,* Irvine, California; and the University of Michigan School of Medicine,† Ann Arbor, Michigan

The nature of the endogenous mediators that down-regulate and curtail the exodus of neutrophils into local acute inflammatory sites is unknown. In the present report, interleukin-6 (IL-6) and transforming growth factor beta (TGFβ), members of a family of macrophage-derived proteins known as cytokines, are shown to inhibit significantly the acute neutrophilic exodus caused by an intratracheal injection of endotoxin (LPS), a proinflammatory component of the cell walls of gram-negative bacteria. Transforming growth factor beta (10 μg) and IL-6 (10 μg) coinjected intratracheally with LPS (10 μg) each inhibited the number of neutrophils in 6-hour bronchoalveolar lavage (BAL) specimens by approximately 50%. The intratracheal coinjection of IL-6, TGFβ, and LPS inhibited the LPS-induced neutrophilic inflammatory exodus by nearly 75%. Interleukin-6 also is shown to be endogenously upregulated within the lung after intratracheal challenge with endotoxin, providing evidence that IL-6 may represent an endogenous negative feedback mechanism to inhibit endotoxin-initiated cytokine-mediated acute inflammation. Interleukin-6 and TGFβ both strongly inhibited the quantity of TNF-α recovered in the BAL fluid of LPS-challenged rats, suggesting that downregulation of LPS-induced TNF-α production within the lung represents one mechanism whereby IL-6 and TGFβ exert an antiinflammatory action. Interleukin-

6 and TGFβ represent novel pharmacologic and, probably, endogenous inhibitors of acute inflammation. (Am J Pathol 1991, 138:1097-1101)

Interleukin-6 (IL-6) and transforming growth factor beta (TGFβ) are multifunctional members of a family of proteins, so called cytokines, that include the proinflammatory factors interleukin-1 (IL-1) and tumor necrosis factor (TNF). Cytokines are released by inflammatory and parenchymal cells in response to endotoxin (LPS), a proinflammatory lipopolysaccharide component of the cell walls of gram-negative bacteria. Cytokines are thought to orchestrate endotoxin-initiated acute inflammation (ie, the migration of neutrophils from blood vessels into tissues). Cytokines also contribute to many of the systemic effects of endotoxemia, such as neutrophilia, lymphopenia, fever, the synthesis of acute-phase proteins by the liver, and hypotensive shock.¹⁻³

Intratracheal injection of endotoxin upregulates IL-1 and TNF expression in the lung and results in severe local acute inflammation.⁴ Intratracheal injection of either IL-1 or TNF also induces acute inflammation in the lung.⁴ Interleukin-1 is a far more potent inducer of acute inflammation than TNF in the lung as well as in other anatomic locations.⁴⁻⁶

The role of IL-6 in local acute inflammation has not, to our knowledge, been defined. Intravenous IL-6, however, was reported by Aderka et al⁷ to decrease intravenous endotoxin-induced serum TNF levels in mice. Our laboratory has confirmed that intravenous IL-6 decreases intravenous endotoxin-induced serum TNF levels in rats.⁸

Supported by NIH award RO1-AI26551.

Accepted for publication March 7, 1991.

Address reprint requests to Dr. Thomas R. Ulich, Department of Pathology, University of California at Irvine, Irvine, CA 91717.

In addition, and perhaps more importantly in regard to the pathogenesis of acute inflammation, we have observed that pretreatment with IL-6 decreases IL-1 mRNA levels in splenic and hepatic whole-organ RNA extracts of endotoxemic rats.⁸ Because IL-6 downregulates endotoxin-induced TNF and IL-1 expression, and because IL-1 and TNF are thought to be proximal mediators of endotoxin-induced inflammation, an experimental animal model was created to test the hypothesis that IL-6 would downregulate LPS-induced acute inflammation and to search for local LPS-induced IL-6 expression.

The role of TGF β in acute inflammation also has not, to our knowledge, been defined. Transforming growth factor beta, similar to IL-6, has been reported to be present in a variety of cell types, including activated macrophages.⁹ Transforming growth factor beta has multiple complex growth stimulatory and growth inhibitory effects on mesenchymal and epithelial cells.¹⁰ It is also a potent immunosuppressive factor. Transforming growth factor beta is present in rheumatoid synovial fluids and can inhibit IL-1-induced lymphocyte proliferation.⁹ It has been reported to suppress the production of TNF α by LPS-stimulated macrophages.¹¹ In light of the known immunosuppressive effects of TGF β , its possible role as an inhibitor of acute neutrophilic inflammation was investigated. Finally, because both IL-6 and TGF β were found to act as pharmacologic inhibitors of local LPS-induced acute inflammation, we tested the anti-inflammatory effect of combined IL-6 and TGF β .

Materials and Methods

Rats (congenic male Lewis rats weighing approximately 225g and anesthetized with ether) were injected intratracheally with equal volumes (0.5 ml) of various doses and combinations of either endotoxin (*S. Typhus* lipopolysaccharide, Sigma Chemical Co., St. Louis, MO), recombinant human IL-6, or recombinant human TGF β_1 (gifts of Dr. Lawrence Souza, Amgen, Inc., Thousand Oaks, CA). Six hours later, the rats were killed and bronchoalveolar lavage (BAL) via the instillation of a tracheal catheter was

performed to enumerate the absolute number of neutrophils in the intra-alveolar inflammatory exudate. The 6-hour timepoint previously has been documented by our laboratory⁴ to represent the peak of the neutrophilic inflammatory exodus. Experiments were performed by randomly dividing rats into experimental groups to be injected on the same day with either LPS or with LPS plus IL-6 or TGF β . A typical day's experiment consisted of injecting three rats intratracheally with LPS alone and three rats intratracheally with LPS plus a cytokine. The absolute number of neutrophils in the BAL specimens of the rats from each experimental group is expressed as the mean plus or minus one standard error of the mean. The probability value was determined by the two-tailed *t*-test for unpaired data. Histologic examination of Bouin's-fixed paraffin-embedded sections of the lungs after BAL was performed to evaluate the accumulation of neutrophils within pulmonary parenchyma and the adequacy of BAL. Tumor necrosis factor protein determinations in BAL fluid were determined as previously described.¹² Whole-lung RNA extraction, Northern blotting, and hybridization for IL-6 mRNA were performed as previously described.^{12,13}

Results

The LPS-induced acute inflammatory exodus was inhibited by an average of 61% in rats receiving 10 μ g IL-6 plus 1 μ g LPS ($n = 7$), as opposed to rats receiving 1 μ g LPS alone ($n = 9$) ($p < 0.0001$, Table 1). The LPS-induced neutrophilic exodus was inhibited by an average of 53% in rats receiving 10 μ g IL-6 plus 10 μ g LPS ($n = 18$), as opposed to rats receiving 10 μ g LPS alone ($n = 18$) ($p < 0.0001$, Table 1). Intratracheal injection of IL-6 alone caused the accumulation of a very small number of neutrophils (less than 10^6 neutrophils/BAL, $n = 2$). Intratracheal injection of saline causes no or at most a very slight accumulation of neutrophils in BAL fluid.^{4,14}

After having demonstrated that IL-6 can act as a pharmacologic downregulator of LPS-induced local acute inflammation, we wondered if there was evidence that host

Table 1. IL-6 and TGF β Inhibit Intratracheal LPS-induced Acute Inflammation

Intratracheal injection	n	Neutrophils $\times 10^{-6}$	Inhibition (%)	P value
LPS (1 μ g)	9	6.36 $\pm$ 0.52		
LPS (1 μ g) + IL-6 (10 μ g)	7	2.45 $\pm$ 0.54	61	0.0001
LPS (10 μ g)	18	20.86 $\pm$ 0.17		
LPS (10 μ g) + IL-6 (10 μ g)	18	9.84 $\pm$ 0.12	53	0.0001
LPS (10 μ g)	6	12.67 $\pm$ 1.10		
LPS (10 μ g) + TGF β (1 μ g)	6	8.22 $\pm$ 1.20	35	0.02
LPS (10 μ g)	14	18.74 $\pm$ 1.06		
LPS (10 μ g) + TGF β (10 μ g)	13	8.55 $\pm$ 0.57	54	0.0001

LPS, Lipopolysaccharide, TGF β , transforming growth factor beta.

IL-6 is upregulated locally after the intratracheal injection of LPS and may therefore act as an endogenous negative feedback mechanism to stop the neutrophilic exodus that is the cellular hallmark of acute inflammation. Interleukin-6 mRNA expression was determined in whole-lung RNA extracts by Northern blotting using a rat IL-6 cDNA probe¹³ at sequential timepoints after the intratracheal or intravenous injections of 100 µg LPS. Intratracheal injection of LPS caused IL-6 mRNA expression beginning at 2 hours and increasing markedly at 4 hours (Figure 1). In a further study of pulmonary IL-6 mRNA expression at later timepoints after intratracheal injection of LPS, IL-6 mRNA expression was found to remain at a very high level at 6 hours with a return to preinjection levels at 12, 24, and 48 hours (data not shown). In contrast to the kinetics of IL-6 expression after the intratracheal injection of LPS, intravenous injection of LPS caused IL-6 mRNA expression to peak at 2 hours with a substantial decrease toward baseline level by 4 hours (Figure 1).

The LPS-induced acute inflammatory exodus was inhibited by an average of 54% in rats receiving 10 µg TGFβ plus 10 µg LPS (n = 13), as opposed to rats receiving 10 µg LPS alone (n = 14) (p < 0.0001, Table 1). The LPS-induced accumulation of neutrophils was inhibited by 35% in rats receiving 1 µg TGFβ plus 10 µg LPS (n = 6), as opposed to rats receiving 10 µg LPS alone (n = 6) (p < 0.02, Table 1). Intratracheal injection of TGFβ alone did not cause a significant influx of neutrophils into the lungs (less than 0.1 × 10⁶ neutrophils/BAL).

The combined effects of IL-6 and TGFβ on LPS-induced acute neutrophilic inflammation then were investigated. All four experimental groups (10 µg LPS alone, 10 µg LPS plus 10 µg IL-6, 10 µg LPS plus 10 µg TGFβ, and 10 µg LPS plus 10 µg IL-6 plus 10 µg TGFβ) were studied concurrently. The combination of IL-6 plus TGFβ inhibited the LPS-induced neutrophilic exodus by 74%, as opposed to 35% inhibition with IL-6 alone and 56% inhibition with TGFβ alone (Table 2). The combined inhibitory effect of IL-6 and TGFβ was significantly greater than the inhibitory effect of either cytokine alone (p < 0.008).

Histologic examination of the lungs in all four experimental groups (n = 2 rats from each group) was performed after BAL to exclude the possibility that the decrease in BAL neutrophils might be due to an increase in neutrophils remaining in the lungs of cytokine-treated rats (as might occur, for example, if IL-6 or TGFβ induced the expression of adhesion molecules that caused the neutrophils to adhere more avidly to pulmonary parenchyma). Double-blind microscopic evaluation of the lungs revealed an indistinguishable histologic appearance in the LPS-plus-cytokine-treated groups. The LPS-alone-treated group was distinguished from the other three groups by a greater number of neutrophils. One may therefore conclude that the degree of cytokine-induced inhibition of acute neutrophilic inflammation as measured by BAL is, if anything, an underestimation of the true amount of inhibition. In none of the experimental groups (not even after intratracheal injection of 10 µg LPS

Figure 1. IL-6 mRNA is not constitutively detected in whole-lung RNA preparations of naive rats (time 0) or 1 hour after either intratracheal or intravenous injections of endotoxin. IL-6 mRNA is detectable in the lung at similar levels at 2 hours after either the intratracheal or intravenous injections of endotoxin. At 4 hours after the intratracheal injection of endotoxin, the level of pulmonary IL-6 mRNA has increased dramatically, whereas at the same timepoint after the intravenous injection of endotoxin the level of IL-6 has almost returned to preinjection level. The 4-hour timepoint marks the beginning of the neutrophilic exodus into alveoli after the intratracheal, but not the intravenous, injection of endotoxin.⁴ The expression of splenic IL-6 mRNA in a whole-organ RNA preparation at 2 hours after the intravenous injection of endotoxin is shown for comparison. All lanes of the Northern blot were loaded with 25 µg whole-organ RNA and hybridized with rat IL-6 cDNA. The rat expresses two major IL-6 mRNA species that code for the same protein.¹³

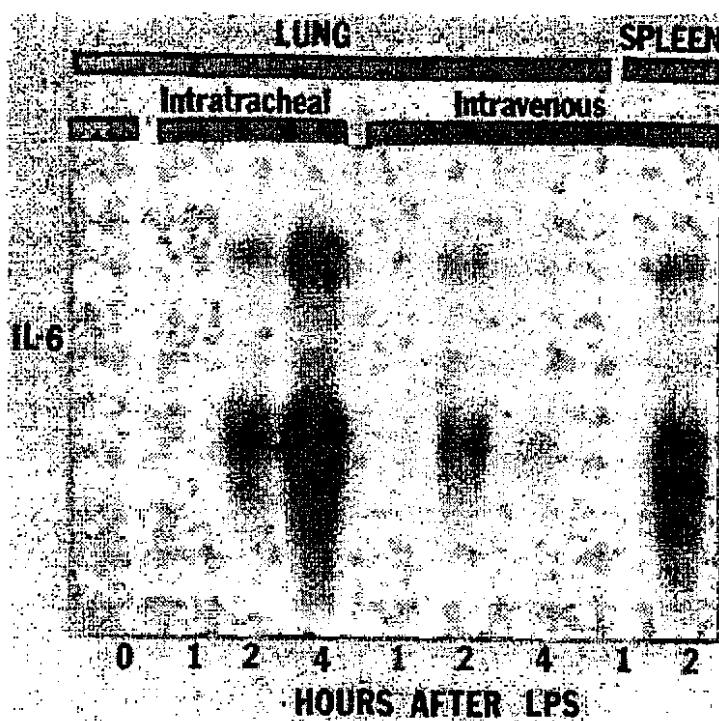


Table 2. *The Combination of IL-6 and TGFβ Inhibits Intratracheal LPS-induced Acute Inflammation More Than Either Cytokine Alone*

Intratracheal injection	n	Neutrophils × 10 ⁻⁶	Inhibition (%)	P value
LPS (10 μg)	8	20.75 ± 1.42	—	—
LPS (10 μg) + IL-6 (10 μg)	8	13.44 ± 1.39	35	0.002
LPS (10 μg) + TGFβ (10 μg)	7	9.10 ± 0.88	56	0.0001
LPS (10 μg) + IL-6 (10 μg) + TGFβ (10 μg)	8	5.50 ± 0.75	74	0.0001

LPS, lipopolysaccharide; TGFβ, transforming growth factor beta.

alone) was any evidence of architectural or cellular injury to the lung observed at the light microscopic level. The LPS-induced inflammatory reaction was histologically characterized by the appearance of a modest number of neutrophils in alveolar septae, alveolar spaces, and in bronchial mucosa, as previously illustrated by our laboratory.⁴ Tumor necrosis factor protein levels in the BAL fluids of the same rats were measured and were found to be decreased by 66% in both LPS-plus-IL-6- and LPS-plus-TGFβ-treated rats and to be decreased by 88% in LPS-plus-IL-6-plus-TGFβ-treated rats as compared to LPS-alone-treated rats (274 ± 30 U TNF/BAL after LPS alone, 94 ± 36 U TNF/BAL after LPS plus IL-6, 96 ± 38 U TNF/BAL after LPS plus TGFβ, and 34 ± 17 U TNF/BAL after LPS plus IL-6 plus TGFβ).

Discussion

Although much new information has emerged during the past few years regarding the acute proinflammatory effects of such cytokines as IL-1 and TNF, relatively little is known about endogenous downregulators of acute inflammation. In the present study, IL-6 and TGFβ, alone and especially in combination, are shown to inhibit strongly LPS-induced acute inflammation. Interleukin-6 and TGFβ thus represent novel antiinflammatory agents of potential therapeutic utility in clinical situations of cytokine-mediated acute inflammation.

Neutrophils first appear in BAL fluids 4 hours after intratracheal injection of endotoxin,⁴ whereas neutrophils are not present in BAL fluid 4 hours after the intravenous injection of endotoxin. The marked increase in pulmonary IL-6 mRNA after intratracheal as opposed to intravenous injection of endotoxin therefore may be, in part, neutrophil derived, especially because Cicco et al¹⁵ recently reported that neutrophils express IL-6 in response to stimulation with endotoxin *in vitro*. The concept that neutrophils may downregulate their own accumulation via the expression of IL-6 is attractive, but our data do not allow us to quantitate the probable increased contribution of alveolar macrophage-derived IL-6 after intratracheal administration as opposed to intravenous administration of endotoxin. Endotoxin-stimulated cells of the monocyte-macrophage lineage are known to secrete large amounts

of IL-6.¹⁶ The amounts of IL-6 and TGFβ that are released into the alveolar fluid of the lung under pathophysiologic conditions are unknown. Interleukin-6 is known, however, to attain concentrations as high as 2 μg/ml in the serum of endotoxemic rats,⁸ suggesting that microgram quantities of endogenous IL-6 also might be attainable in the lung during gram-negative pneumonia.

In conclusion, pharmacologic administration of exogenous recombinant IL-6 and TGFβ inhibits endotoxin-induced local acute inflammation. The mechanism of the anti-inflammatory action of IL-6 and TGFβ may relate to the ability of these cytokines to inhibit TNFα^{8,11} (and as shown by the present data) and IL-1 production⁹ by macrophages. Host-derived IL-6 is upregulated locally after challenge with LPS and may act as an endogenous-negative feedback mechanism to inhibit the LPS-initiated IL-1- and TNF-mediated acute inflammatory process. Finally host macrophage-derived TGFβ may be speculated also to play a role as an endogenous inhibitor of acute inflammation. Transforming growth factor beta has been reported by Roberts et al¹⁷ to increase collagen synthesis *in vitro* and to cause fibrosis *in vivo* after intradermal injection. Transforming growth factor beta is known to be a pleiotropic mediator and may be hypothesized to perform a dual role by acting both to resolve acute inflammation and to begin tissue repair and remodeling by initiating collagen deposition.

Acknowledgments

The authors thank Dr. Lawrence Souza and Dr. Keith Wescott (Amgen, Inc., Thousand Oaks, CA) for providing the recombinant human IL-6 and TGFβ and Drs. W. Northemann and G. Fey (Scripps Research Foundation, La Jolla, CA) for providing rat IL-6 cDNA.

References

1. Sporn MB, Roberts AB: Peptide growth factors are multifunctional. *Nature* 1988; 332:217
2. Ulich TR, del Castillo J, Keys M, Granger G, Ni RX: Kinetics and mechanisms of interleukin-1 and tumor necrosis factor induced changes in circulating numbers of neutrophils and lymphocytes. *J Immunol* 1987; 139:3406-3415

3. Cybulsky ME, Chan MK, Movat HZ: Acute inflammation and microthrombosis induced by endotoxin, IL-1, and TNF and their implication in gram-negative infection. *Lab Invest* 1988, 58:365-378
4. Ulich TR, Watson LR, Yin S, Guo K, del Castillo J: The intratracheal administration of endotoxin and cytokines I: Characterization of LPS-induced TNF and IL-1 mRNA expression and the LPS-, TNF-, and IL-1-induced inflammatory infiltrate. *Am J Pathol* (In press)
5. Wankowicz Z, Megyeri P, Issekutz A: Synergy between TNF and IL-1 in the induction of polymorphonuclear leukocyte migration during inflammation. *J Leuk Biol* 1988, 43:349-356
6. Sayers TJ, Wiltrout TA, Bull CA, Denn AC, Pilaro AM, Lokesh B: Effects of cytokines on polymorphonuclear infiltration in the mouse. *J Immunol* 1988, 141:1670-1677
7. Aderka D, Le J, Vitcek J: IL-6 inhibits lipopolysaccharide-induced TNF production in cultured human monocytes, U937 cells, and in mice. *J Immunol* 1989, 143:3517
8. Ulich TR, del Castillo J, Remick D, Guo K, Yin S, Weng P: Endotoxin-induced cytokine gene expression *in vivo* III: Interleukin-6 mRNA and serum protein expression and the *in vivo* hematologic effects of IL-6. *J Immunol* (In press)
9. Wahl SM, Allen JB, Wong HL, Dougherty SF, Ellingsworth LR: Antagonistic and agonistic effects of TGF β and IL-1 in rheumatoid synovium. *J Immunol* 1990, 145:2514-2519
10. Moses HL, Yang EY, Pieterpol JA: TGF β stimulation and inhibition of cell proliferation: New mechanistic insights. *Cell* 1990, 63:245-247
11. Dunham DM, Arkins S, Edwards CK, Dantzer R, Kelley KW: Role of interferon- γ in counteracting the suppressive effects of TGF β 2 and glucocorticoids on the production of TNF α . *J Leuk Biol* 1990, 48:473-481
12. Ulich TR, Guo K, Irwin B, Remick DG, Davatellis GN: Endotoxin-induced cytokine gene expression *in vivo* II. Regulation of TNF and IL-1 expression and suppression. *Am J Pathol* 1990, 137:1137-1185
13. Northemann W, Bracial TA, Hattori M, Lee F, Fay GH: Structure of the rat IL-6 gene and its expression in macrophage-derived cells. *J Biol Chem* 1990, 264:16072
14. Ulich TR, Yin S, Guo K, del Castillo J, Eisenberg SP, Thompson RC: The intratracheal administration of endotoxin and cytokines III. The IL-1 receptor antagonist inhibits endotoxin- and IL-1-induced acute inflammation. *Am J Pathol* 1991, 138:521-524
15. Cicco NA, Lindemann A, Content J, Vandebussche P, Lubbert M, Gauss J, Mertelsmann R, Herrman F: Inducible production of IL-6 by human polymorphonuclear neutrophils: Role of GM-CSF and TNF. *Blood* 1990; 75:2049
16. Kato K, Yokoi T, Takano N, Kanegane H, Yachie A, Miyawaki T, Taniguchi N: Detection by *in situ* hybridization and phenotypic characterization of cells expressing IL-6 mRNA in human stimulated blood. *J Immunol* 1990, 144:1317
17. Roberts AB, Sporn MB, Assoian RK, Smith JM, Roche NS, Wakefield LM, Heine UI, Liotta LA, Falanga V, Keri JH, Fauci AS: TGF β : Rapid induction of fibrosis and angiogenesis *in vivo* and stimulation of collagen synthesis *in vitro*. *PNAS USA* 1986, 83:4167-4171

COMBINATION OF METHOTREXATE AND PREDNIZONE DECREASES CIRCULATING CONCENTRATIONS OF INTERLEUKIN 1 β AND INTERLEUKIN 6 IN PATIENTS WITH RHEUMATOID ARTHRITIS. POOR CORRELATION OF CYTOKINE SUPPRESSION WITH CLINICAL IMPROVEMENT.

D. NOWAK*, J. LEWANDOWICZ*, B. DBKOWSKA* and J. MARCZAK*

*Department of Pneumology and Allergy, Medical University of Lodz, 90-153 Lodz, Kopcińskiego 22, Poland. * Division of Rheumatology, Medical University of Lodz, 94-029 Lodz, Minska 2, Poland

Received September 8, 1998 - Accepted November 11, 1998

Objective - To evaluate the effect of methotrexate (MTX) in combination with prednisone on cytokine levels, acute phase proteins and thiobarbituric acid reactive substances (TBAR - an indicator of peroxidative damage to tissue lipids) in the blood of rheumatoid arthritis (RA) patients and to investigate their associations with clinical disease activity.

Methods - We measured blood concentrations of interleukin-1 β (IL-1 β), interleukin-6 (IL-6), TBARs and classical clinical and laboratory indices of disease activity in 36 RA subjects before and after 3 and 6 month treatment with MTX and prednisone. Only RA subjects who stopped any disease-modifying anti-rheumatic drugs treatment for last 3 months were included in the study. Baseline cytokine and TBARs levels were compared with those obtained with 20 healthy controls.

Results - Compared to controls RA subjects had elevated levels of circulating IL-1 β (63.3 ± 47.6 vs 13.7 ± 7.8 pg/ml, $p < 0.01$), IL-6 (147.2 ± 76.5 vs 15.9 ± 13.3 pg/ml, $p < 0.001$) and TBARs (3.11 ± 0.42 vs 1.34 ± 0.45 mmol/l, $p < 0.001$) concentrations. MTX in combination with prednisone improved patient clinical status that was accompanied by 1.96-, 1.25-, and 1.35-fold decrease in IL-1 β , IL-6 and TBARs after 6 month treatment ($p < 0.001$), respectively. Although, IL-1 and IL-6 revealed a few correlations with classical indices of disease activity no association was found between patient clinical status improvement and cytokine changes over 6 month treatment.

Conclusions - MTX in combination with prednisone decreases blood levels of IL-1 β and IL-6 and inhibits the intensity of free radical-mediated processes in RA subjects. Monitoring of plasma concentrations of these cytokines could not predict the treatment efficacy.

Rheumatoid arthritis (RA) is a systemic inflammatory disease characterized by enhanced production and increased serum levels of acute phase proteins and proinflammatory cytokines that may reflect the disease activity. The inflamed processes are preferentially localized in synovial joints and inflamed synovium is an important source of interleukin-1 β (IL-1 β) and interleukin-6 (IL-6) (1,2). Circulating IL-6 has been shown to correlate with some clinical measures of disease activity (3,4,5) and suppression of blood IL-6

levels correlated with decrease in the number of inflamed joints and morning stiffness as outcome of a long term administration of disease-modifying anti-rheumatic drugs (DMARDs) (5). There was also an association between the levels of this cytokine and the local disease activity and IL-6 has been shown to be indicator of proinflammatory and potentially erosive activity in synovial fluid of RA patients (2,6). On the other hand, blood levels of IL-6 did not reveal any association with radiological progression of joint damage during

Key words: Methotrexate, prednisone, cytokines, rheumatoid arthritis, thiobarbituric acid reactive substances

Mailbox address: Dariusz Nowak,
Department of Pneumology and Allergy,
Medical University of Lodz, 90-153 Lodz,
Kopcińskiego 22, Poland

0394-6320 (1999)
Copyright © by BILIMEDICAL RESEARCH PUBLISHING, Ltd.
This publication and/or article is for individual use only and may not be further
reproduced without written permission from the copyright holder.
Unauthorized reproduction may result in financial and other penalties.

three year observation of patients with early RA (7). Although, peripheral blood mononuclear cells (PBMC) of RA patients release more IL-1 β than cells of healthy subjects (8) the circulating levels of IL-1 β in the course of RA seems to be not elevated (9,10). However, these RA patients were under treatment with DMARDs introduced a few months before entering the study. Therefore, the drug -induced IL-1 β suppression can not be excluded. Methotrexate (MTX), that belongs to DMARDs is increasingly used in the treatment of RA (11,12,13). Weekly low dose treatment with MTX alone or in combination with sulphasalazine was highly effective and caused the decrease in the circulating concentrations of IL-6 (8,14). This was accompanied by suppression of IL-1 β release from PBMC (8) and the decrease in IL-1 β concentrations in synovial fluid (1,15). The combination of MTX with prednisone is one of those used in the treatment of RA (10). Prednisone inhibits majority of inflammatory cells that are able to synthesise and release IL-1 β and IL-6 (16,17). It is possible that administration of MTX and prednisone may suppress the circulating concentrations of these cytokines in RA subjects. Scarry data are available on efficacy of MTX in combination with prednisone and correlations between treatment -induced changes in IL-6 and IL-1 β levels and the attenuation of disease activity in RA subjects (10,12). Therefore, in this study we evaluated the effect of low oral dose of MTX in combination with prednisone on circulating concentrations of IL-6 and IL-1 β in relation to changes in clinical and laboratory parameters in patients with active RA who passed the 3 month DMARDs washout period.

MATERIALS AND METHODS

Patients and study protocol

Thirty six subjects (mean age 58 ± 10 years, 28 women, 8 men) who fulfilled the American College Rheumatology criteria for definite or classical RA, (18) and 20 healthy subjects who had not suffered from any infectious disease for the previous four months were studied. Healthy controls (mean age 56 ± 6 years, 15 women, 5 men) members of our hospital staff were free of any medications and routine physical examination showed nothing abnormal. RA patients (mean disease duration = 8 ± 6 years, rheumatoid factor $\pm = 28/8$) had

active disease, defined as ESR > 30 mm, morning stiffness > 60 min, number of swollen joints > 6 and number of tender joints > 9 (Table 1). They were selected from all consecutive patients who attended our department in a period of 15 months. Since, piroxicam has been reported to have no influence on MTX pharmacokinetics and serum IL-6 and IL-1 β levels (10,19), only RA subjects who used this non-steroidal, anti-inflammatory drug were included in the study. Twenty nine of RA patients were treated with at least one of DMARDs (injectable gold, hydroxychloroquine, D-penicillamine, sulphasalazine or azathioprine) in the past. Three subjects received 4 of this drugs, 5 subjects were treated with 3 DMARDs, and 12 and 9 patients used 2 and 1 of DMARDs, respectively. The medication with any DMARDs was stopped at least 3 months before the study. All patients were allocated to 6 month therapy with MTX (7.5 mg weekly in two separate doses: 5 mg in the first and 2.5 mg in the second day of week), oral prednisone (7.5 mg daily) and folic acid (15 mg daily, except days in which MTX was used). Piroxicam as a concomitant treatment was kept at a stable dose (20 mg a day) during the study. Blood for laboratory determinations was drawn before and after 3 and 6 months of treatment. This was accompanied by assessment of swollen and painful joints, morning stiffness, grip strenght (left and right, measured with hand dynamometer) and determination of Ritchie articular index (maximum score = 78). The study had the approval of the local Ethics Committee, and informed consent was obtained.

Circulating cytokine concentrations

Blood samples (7 ml) for cytokine determinations were drawn in Vacutainer tubes (Becton & Dickinson USA, containing 48 μ l EDTA-K3) at the same time of day (8-10 a.m.). After 30 min incubation at room temperature they were centrifuged and obtained plasma was frozen at -80°C and stored not more than 30 days until assay. IL-1 β and IL-6 concentrations were measured with IL-1 β -IRMA and IL-6-IRMA (immunoradiometric assay Medgenix Diagnostics, Fleurus Belgium code 30 121 20 and 30 126 00) according to manufacturers instructions. The calibration curve was prepared with appropriate cytokine standard solutions before each series of measurements. The radioactivity related to signal antibodies labelled with I-125 was counted with liquid scintillation analyzer (TRI CARB 1600 TR Packard) set in gamma mode. The sensitivity of IL-1 β and IL-6 detection was 5 and 6 pg/ml, respectively.

Other determinations

The plasma levels of acute phase proteins (haptoglobin, alpha-1-proteinase inhibitor), immunoglobulins (IgG, IgM, IgA) and complement C3 and C4 fractions were determined using a rate nephelometric assay (Beckman Array 360 system). All individual measurements were carried out in duplicates and mean values were calculated. The plasma concentration of thiobarbituric acid reactive substances (TBARs) was measured spectrofluorimetrically as previously described (20). ESR, full blood cell count (Technicon, Belgium) and seromucoid plasma concentrations were done by a central laboratory of Medical University Hospital.

Statistical analysis

Results are expressed as mean $\pm$ standard deviation. Paired data were analyzed by a Wilcoxon matched pairs test. For non-paired samples (baseline values in RA subjects and in healthy controls) Student t-test and Kolmogorov-Smirnov test were used depending on sample distribution. Correlations were calculated by means of the Spearman rank order correlation. A p-value of less than 0.05 was considered to be significant.

RESULTS

Clinical results

Six month treatment with MTX and prednisone resulted in the improvement in RA subjects (Table 1). The Ritchie articular index decreased by about two-fold from 25.6 to 12.1 ($p < 0.05$). It was accompanied by the decrease in the number of swollen joints and morning stiffness as well as the rise in the grip strength. The normalization of ESR and the increase in the haematocrit and haemoglobin level was also observed. No patient required increase in the MTX and/or prednisone daily dose during participation in this six month open study. Combination of MTX with prednisone was relatively well tolerated. The following side-effects, nausea, stomatitis, and rash were observed in 4, 2 and 1 subjects, respectively. The number of platelets decreased significantly at the end of study (Table 2), however, this value was still within normal range and no bleeding disturbances were noted. No patient dropped out due to these adverse drug reactions. Clinical improvement

was accompanied by the significant decrease in ESR, plasma levels of C3 and C4 complement fractions, seromucoid and acute phase proteins (Table 2). Most of these variables changed significantly just after 3 month treatment. However, plasma IgM level did not change in response to treatment with MTX and prednisone. No differences in response to the treatment were observed in RA subjects with detectable and not detectable rheumatoid factor (data not shown). The results of this small open study suggest that combination of MTX and prednisone is effective in the treatment of RA subjects with active disease.

Effect of treatment on circulating cytokine and TBARs concentration

The mean levels of circulating IL-6 and IL-1 β were significantly higher in RA patients than in healthy subjects (147.2 ± 76.5 vs 15.9 ± 13.3 pg/ml, $p < 0.0001$ and 63.3 ± 47.6 vs 13.7 ± 7.8 pg/ml, $p < 0.01$, respectively). Similarly, plasma concentration of TBARs was 2.3-fold higher in RA group than in healthy controls (3.11 ± 0.42 vs 1.34 ± 0.45 nmol/ml, $p < 0.0001$). Treatment with MTX and prednisone caused the significant decrease in the plasma levels of both analysed cytokines (Table 3). The mean decline in IL-6 concentration was 15.1 ± 19.6 pg/ml at 3 month and 29.7 ± 21.8 pg/ml at 6 month and correlated positively with its initial (before treatment) concentration, $r = 0.47$ ($p < 0.004$) and $r = 0.53$ ($p < 0.0007$), respectively. For IL-1 β the mean decrease was 26.8 ± 30.9 pg/ml at 3rd month and 31.0 ± 52.7 pg/ml at the end of the study and this also correlated positively with baseline IL-1 β level, $r = 0.78$ ($p < 0.00001$) and $r = 0.69$ ($p < 0.00001$). The concentrations of IL-1 β measured before and after 3 and 6 month treatment did not correlate with IL-6 levels, $r = 0.13$, $r = 0.03$ and $r = 0.15$, respectively. There was also no association between treatment-induced decrease in both cytokines, either after 3 month ($r = 0.01$) and 6 month treatment ($r = 0.03$). The suppression of circulating IL-1 β and IL-6 levels was accompanied by the decrease in plasma concentration of TBARs. The level of TBARs at the end of treatment was 1.35-fold lower than that at baseline. Additional measurements revealed significant suppression of circulating TBARs generation just after 6 weeks of treatment (Table 3).

variable	treatment		p <
	before	after	
Ritchie articular index	25.6 ± 6.6	12.1 ± 4.1	0.05
Number of swollen joints	14 ± 6	6 ± 3	0.001
Grip strength right (mmHg)	90 ± 21	148 ± 27	0.001
Grip strength left (mmHg)	88 ± 17	147 ± 25	0.001
morning stiffness (min)	119 ± 28	47 ± 18	0.001

Table I. Clinical parameters before and after 6 month treatment of RA patients with combination of methotrexate and prednisone.

Table II. Laboratory parameters at baseline and after 3 and 6 month treatment with methotrexate and prednisone.

variable	before	after treatment			
		3 month	p <	6 month	p <
ESR (mm)	79 ± 30	39 ± 21	0.05	27 ± 11	0.05
Haematocrit (%)	35.1 ± 3.1	36.8 ± 2.2	0.02	38.2 ± 1.9	0.002
Haemoglobin (g/dl)	10.9 ± 0.9	11.4 ± 0.7	0.002	11.9 ± 0.6	0.0001
WBC (cells x 10 ³ /μl)	7.02 ± 2.01	7.20 ± 1.67	ns	7.30 ± 1.14	ns
platelets (cells x 10 ³ /μl)	256 ± 45	253 ± 31	ns	220 ± 30	0.0007
haptoglobin (g/l)	3.02 ± 1.14	2.50 ± 1.21	0.05	2.41 ± 0.79	0.05
transferrin (g/l)	2.64 ± 0.74	2.33 ± 0.76	0.05	2.15 ± 0.62	0.05
alpha-1-proteinase inhibitor (g/l)	3.03 ± 0.89	2.56 ± 0.85	0.05	2.41 ± 0.79	0.05
IgG (g/l)	13.03 ± 3.91	10.6 ± 3.87	0.05	9.64 ± 3.49	0.05
IgA (g/l)	3.43 ± 1.57	2.63 ± 1.21	0.05	2.53 ± 1.23	0.05
IgM (g/l)	2.08 ± 1.27	2.20 ± 1.60	ns	1.83 ± 0.78	ns
C3 (mg/dl)	139 ± 42	120 ± 41	0.05	110 ± 34	0.05
C4 (mg/dl)	39 ± 13	35 ± 13	ns	32 ± 11	0.05
serumucoid (mg/l)	181.2 ± 56.9	129.6 ± 38.7	0.001	105.9 ± 20.4	0.001

ESR = erythrocyte sedimentation rate, WBC = white blood cells

Table III. Plasma concentrations of IL-1 β , IL-6 and thiobarbituric acid reactive substances (TBARs) before and after 3 and 6 month treatment with methotrexate and prednisone.

variable	before	after treatment			
		3 months	p <	6 months	p <
IL-1 β (pg/ml)	63.3 $\pm$ 47.6	36.4 $\pm$ 36.5	0.001	32.3 $\pm$ 60.6	0.001
IL-6 (pg/ml)	147.2 $\pm$ 76.5	132.2 $\pm$ 96.8	0.001	117.6 $\pm$ 109.4	0.001
TBARs (nmol/ml)	3.11 $\pm$ 0.42	2.58 $\pm$ 0.67	0.001	2.31 $\pm$ 0.57	0.001

TBARs measured additionally at 6- and 18th week of treatment were 2.57 $\pm$ 0.67 and 2.50 $\pm$ 0.58 nmol/ml and were lower than those at baseline (p< 0.001).

Table IV. Correlations (r) between three and six month treatment - induced decrease in clinical and laboratory parameters and their initial (before treatment) values.

variable	decrease after 3 months	r	p<	decrease after 6 months	r	p<
IL-1 β (pg/ml)	26.8 $\pm$ 30.9	0.78	0.00001	31.0 $\pm$ 52.7	0.69	0.00001
IL-6 (pg/ml)	15.1 $\pm$ 19.6	0.47	0.004	29.7 $\pm$ 21.8	0.53	0.0007
TBARs (nmol/ml)	0.52 $\pm$ 0.52	0.42	0.02	0.80 $\pm$ 0.57	0.39	0.03
a1PI (g/l)	0.48 $\pm$ 0.85	0.63	0.0005	0.62 $\pm$ 0.82	0.55	0.003
HPT (g/l)	0.51 $\pm$ 0.91	0.36	ns	0.50 $\pm$ 0.99	0.49	0.009
SMD (mg/dl)	51.6 $\pm$ 35.5	0.76	0.00001	57.2 $\pm$ 32.7	0.79	0.00001
TR (g/l)	0.31 $\pm$ 0.52	0.40	0.04	0.49 $\pm$ 0.52	0.64	0.0004
C3 (mg/dl)	19 $\pm$ 44	0.51	0.007	29 $\pm$ 44	0.68	0.0001
C4 (mg/dl)	4 $\pm$ 13	0.47	0.02	6 $\pm$ 15	0.73	0.0001
IgG (g/l)	2.43 $\pm$ 2.20	0.32	ns	3.40 $\pm$ 3.05	0.58	0.002
IgA (g/l)	0.80 $\pm$ 0.84	0.47	0.02	0.90 $\pm$ 0.86	0.56	0.004
MS (min)	39 $\pm$ 19	0.73	0.00001	72 $\pm$ 24	0.74	0.00001
ESR (mm)	40 $\pm$ 29	0.78	0.00001	52 $\pm$ 28	0.94	0.00001
NSJ	ND	ND	ND	7 $\pm$ 4	0.76	0.0001
RAI	ND	ND	ND	13.5 $\pm$ 4.8	0.75	0.00001

Thirty six RA subjects were treated with low doses of methotrexate and prednisone for 6 months. The changes in clinical and laboratory parameters were evaluated after 3 and 6 month treatment and their correlations with initial (before treatment) values were analysed.

a1PI - alpha-1-proteinase inhibitor, HPT - haptoglobin, SMD - seromucoid, TR - transferrine, MS - morning stiffness, NSJ - number of swollen joints, RAI - Ritchie articular index.

The material on this page was copied from the collection of the National Library of Medicine by a third party and may be protected by U.S. Copyright law.

Table V. Correlations (r) between cytokine (IL-1 β , IL-6) levels and clinical and biochemical parameters before and after treatment with MTX and prednisone in RA subjects.

variable	before treatment		after treatment			
	IL-1 β	IL-6	3 months		6 months	
			IL-1 β	IL-6	IL-1 β	IL-6
MS	0.17	0.13	-0.05	0.05	0.25	-0.04
NSJ	-0.02	-0.16	ND	ND	-0.06	0.11
RAI	-0.26	-0.10	ND	ND	0.09	-0.14
GS left	0.09	-0.34 *	ND	ND	0.09	-0.17
GS right	0.04	-0.39**	ND	ND	-0.02	-0.27
ESR	-0.21	-0.14	0.10	-0.07	0.08	0.03
aPI	0.32	0.11	0.21	0.39*	0.47**	0.45**
HPT	0.19	0.28	-0.16	0.40*	-0.12	0.33
TBARs	0.13	-0.03	0.05	-0.33*	0.17	-0.09
TR	0.22	0.07	0.18	0.40*	0.42*	0.46**
SMD	-0.02	-0.09	-0.03	-0.12	-0.01	-0.02
PLT	-0.44 ***	0.09	-0.06	0.06	0.05	-0.07
Hb	0.24	-0.01	0.16	0.06	-0.13	0.14

Thirty six RA subjects were treated with low doses of methotrexate and prednisone for 6 months. The plasma concentrations of IL-1 β and IL-6 were determined before and after 3 and 6 month treatment. The correlations between cytokine levels and selected clinical and laboratory parameters were evaluated. MS - morning stiffness, NSJ - number of swollen joints, RAI - Ritchie articular index, GS - grip strength, aPI - alpha-1-proteinase inhibitor, HPT - haptoglobin, TR - transferrine, SMD - seromucoid, PLT - platelets, Hb - haemoglobin. Significant correlations : * - $p < 0.05$, ** - $p < 0.02$, *** - $p < 0.01$.

Correlations between cytokine concentrations and clinical and laboratory parameters
Almost all clinical and laboratory parameters improved over the treatment with MTX and prednisone (Table 1, 2 and 3). The higher was baseline value the higher treatment-induced decline was noted after 3 and 6 month drug administration, respectively. This association was highest ($r > 0.70$) for ESR, IL-1 β , seromucoid, C4, morning stiffness, Ritchie articular index and number of swollen joints (Table 4). However, analysis of correlations between circulating cytokine levels and other clinical and laboratory variables showed

only few statistically significant results. Baseline blood IL-1 β concentrations correlated inversely with platelet number ($r = -0.44$, $p < 0.01$). IL-1 β did not correlate with any analysed clinical and laboratory variable after 3 month treatment (Table 5). At the end of study (after 6 month treatment) circulating IL-1 β correlated with serum concentrations of alpha-1-proteinase inhibitor ($r = 0.47$, $p < 0.02$) and transferrine ($r = 0.42$, $p < 0.05$). More associations were found for IL-6. Blood levels of IL-6 correlated inversely with left and right grip strength ($r = -0.34$, $p < 0.05$ and $r = -0.39$, $p < 0.02$) at the start of study and also with

The material on this page was copied from the collection of the National Library of Medicine by a third party and may be protected by U.S. Copyright law.

plasma levels of TBARs, alpha-1-proteinase inhibitor, transferrin and haptoglobin after 3 month treatment.

After 6 month treatment only moderate correlation with this cytokine and alpha-1-proteinase inhibitor and haptoglobin plasma concentrations was found (Table 5). Although treatment-induced suppression of disease activity was accompanied by the decrease in blood cytokine levels no correlations between the decline in IL-6 concentrations and all clinical parameters of disease activity were found. Moreover, suppression of IL-6 did not correlate with changes of ESR and plasma concentrations of seromucoid, haptoglobin, alpha-1-proteinase inhibitor, TBARs and immunoglobulins either after three and six month treatment (data not shown). The same was observed for the decrease in IL-1 β concentrations apart from significant association with the decrease in transferrin and haptoglobin plasma concentrations ($r = 0.57$, $p < 0.002$, $r = 0.39$, $p < 0.05$) after 3 month treatment and the decrease in Ritchie articular index at the end of study.

DISCUSSION

In this open study we found that combination of low doses of MTX and oral prednisone decreases the disease activity in RA subjects over six month period. This treatment was well tolerated and the positive clinical response was accompanied by suppression of classical markers of inflammation and circulatory levels of IL-1 β , IL-6 and TBARs. The baseline cytokine concentrations and their changes over treatment did not correlate with patient's clinical status improvement and majority of indices of inflammation. Previous studies showed that MTX alone or in combination with sulphasalazine decreases the elevated concentrations of circulatory levels of IL-6 in RA subjects (5,8,12,21). IL-6 measurements correlated only with baseline CRP and ESR (8) and no associations between treatment-induced changes in IL-6 and clinical parameters were reported (8,21). This is consistent with study of Leuven and coworkers in which circulating IL-6 correlated with ESR, serum CRP and amyloid while time integrated IL-6 values revealed no significant correlation with radiological progression in subjects with early RA over three years follow up (7). Recent study,

however, showed that decrease in circulating IL-6 during one year administration of MTX correlated with clinical improvement expressed as changes in morning stiffness and number of inflamed joints after three year treatment (5). On the other hand, IL-6 suppression did not correlate with three year changes of joint pain and number of bony erosions (5). The baseline grip strength was the only one clinical parameter that correlated inversely with IL-6 readings in our study. This indicates that measurement of baseline and treatment-induced changes in circulating IL-6 concentration can not predict the efficiency of six month treatment with MTX and prednisone in RA subjects.

We observed elevated IL-1 β concentrations that decreased after administration of MTX with prednisone.

This is different from previous studies showing low or undetectable IL-1 β concentrations in RA subjects (5,8,9,10). Our patients had stopped any medication with DMARDs for three month before entering the study while subjects involved in referred trials had stable treatment with DMARDs and/or with prednisone initiated several months before starting the study (5,8,9,10). It may explain this discrepancies since DMARDs have been reported to decrease circulating IL-1 β levels and to inhibit IL-1 β synthesis by PBMC (22,23). The inhibitory effect of MTX on blood levels of IL-1 β remains open question (11,12).

Some authors reported no changes of circulating IL-1 β levels (8,9) while others showed suppression of IL-1 β in blood and synovial fluid in response to MTX administration (1,15,24). Glucocorticosteroids strongly inhibit functional activation of macrophages (16,17) that are the main source of IL-1 β in rheumatoid synovium (1,25). Therefore, administration of MTX and prednisone in RA subjects who passed 3 month DMARDs washout period resulted in significant suppression of circulating IL-1 β concentrations.

No associations between blood levels of IL-1 β and clinical measures of disease activity suggest that monitoring of circulating IL-1 β concentrations has low and even no prognostic value.

The production of IL-6 by synoviocytes is enhanced by IL-1 (26) and there is a positive correlation between concentrations of these cytokines in rheumatoid synovial fluid (2).

Although, we found that circulating IL-1 β and IL-6 were higher than in healthy controls, surprisingly there was no associations between baseline levels of these cytokines and the treatment-induced suppression. The stimulatory effect of other factors (eg. TNF) on IL-6 synthesis (26) and the ability of IL-6 to inhibit IL-1 β and TNF production (27) may be possible explanation of this negative results.

Elevated blood TBARs suggest the occurrence of enhanced lipid peroxidation in RA subjects. TBARs could also be formed during oxidative injury of DNA, proteins and/or carbohydrates (28). Hence, elevated TBARs levels may reflect the increased intensity of peroxidative damage to tissues of the joint. Polymorphonuclear leukocytes, monocytes and macrophages could be the source of free radicals responsible for TBARs generation in RA subjects (29). These cells are primed by cytokines including IL-1 β and TNF to produce more reactive oxygen species (17). The inhibitory effect of MTX and prednisone on phagocytes (11,12,17) may explain the decrease of blood levels of TBARs just after 6 weeks of treatment in RA subjects. IL-1 β can prime phagocytes to produce more reactive oxygen species, however, we did not observe any association between suppression of circulating TBARs and IL-1 β . It is possible that blood TBARs may represent only small part of end products of lipid peroxidation that diffuse from inflamed joints and are independent on the circulating IL-1 β . However, these results clearly show that MTX in combination with prednisone reduces the intensity of harmful free-radical mediated processes in subjects with active RA.

In conclusion we found that RA subjects who passed 3 month DMARDs washout period had elevated circulatory IL-1 β , IL-6 and TBARs levels that decrease after treatment with MTX and prednisone. However treatment-induced suppression of these parameters did not correlate with patient clinical status improvement and could not be useful for prediction of the treatment efficacy.

REFERENCES

1. Dolhain R.J.E.M., P.P. Tak, B.A.C. Dijkmans, P. De Kuiper, P.C. Breedveld and A.M.M. Miltenburg. 1998. Methotrexate reduces inflammatory cell numbers, expression of monokines and of adhesion molecules in synovial tissue of patients with rheumatoid arthritis. *Br. J. Rheumatol.* 37:502.
2. Leeuwen M.A., J. Westra, P.C. Limburg, P.L.C.M. Riel, and M.H. Rijswijk. 1995. Interleukin-6 in relation to other proinflammatory cytokines, chemotactic activity and neutrophil activation in rheumatoid synovial fluid. *Ann. Rheum. Dis.* 54:33.
3. Madhok R., A. Crilly, J. Watson and H.A. Capell. 1993. Serum interleukin-6 levels in rheumatoid arthritis. Correlations with clinical and laboratory indices of disease activity. *Ann. Rheum. Dis.* 52:232.
4. Dasgupta B., M. Corkill, B. Kirkham, T. Gibson and G. Panayi. 1992. Serial estimation of interleukin 6 as a measure of systemic disease in rheumatoid arthritis. *J. Rheumatol.* 19:22.
5. Straub R.H., U. Muller-Jadlow, T. Lichtinger, J. Scholmerich, H. Menninger and D. Lang. 1997. Decrease of interleukin 6 during the first 12 months is a prognostic marker for clinical outcome during 36 months treatment with disease-modifying anti-rheumatic drugs. *Br. J. Rheumatol.* 36:1298.
6. Miltenburg A.M.M., J.M. Van Laar, R. de Kuiper, M.R. Dahn and P.C. Breedveld. Interleukin-6 activity in paired samples of synovial fluid. Correlation of synovial fluid interleukin-6 levels with clinical and laboratory parameters of inflammation. *Br. J. Rheumatol.* 30:186.
7. Leeuwen M.A., J. Westra, P.C. Limburg P.C., P.L.C.M. Riel and M.H. Rijswijk. 1995. Clinical significance of interleukin-6 measurement in early rheumatoid arthritis: relation with laboratory and clinical variables and radiological progression in a three year prospective study. *Ann. Rheum. Dis.* 54:674.
8. Barrera P., C.J. Hongsma, A.M.T.H. Boerbooms, P.L.C.M. Riel, G.F. Borm, L.J.A. Putte, J.W.M. Meer. 1995. Effect of methotrexate alone or in combination with sulphasalazine on the production and circulating concentrations of cytokines and their antagonists. Longitudinal evaluation in patients with rheumatoid arthritis. *Br. J. Rheumatol.* 34:747.
9. Barrera P., A.M.T.H. Boerbooms, P.N.M. Demacker, L.J.A. Putte, H. Gullit, J.W.M. Van der Meer. 1994. Circulating concentrations and production of cytokines and soluble receptors in rheumatoid arthritis patients: effects of a single dose methotrexate. *Br. J. Rheumatol.* 33:1017.
10. Littman B.H., C.E. Drury, R.O. Zimmerman, C.B. Stack and C.C. Law. 1995. Rheumatoid arthritis treated with tenidap and piroxicam. Clinical associations with cytokine modulation by tenidap. *Arthritis Rheum.* 38:29.
11. Kremer J.M. 1995. Possible mechanisms of action of methotrexate in patients with rheumatoid arthritis. *Br. J. Rheumatol.* 34(suppl. 2):26.
12. Furst D.E. 1997. The rational use of methotrexate in rheumatoid arthritis and other rheumatic diseases. *Br. J. Rheumatol.* 36:1196.
13. Rau R., G. Herborn, H. Menninger and J. Blechschmidt. 1997. Comparison of intramuscular methotrexate and gold sodium thiomalate in the treatment of early erosive rheumatoid arthritis: 12 month data of a double-blind parallel study of 174 patients. *Br. J. Rheumatol.* 36:345.

1. Dolhain R.J.E.M., P.P. Tak, B.A.C. Dijkmans, P. De Kuiper, P.C. Breedveld and A.M.M. Miltenburg. 1998. Methotrexate reduces inflammatory cell numbers, expression of monokines and of adhesion molecules in synovial tissue of patients with rheumatoid arthritis. *Br.*

14. Haagsma C.J., P.L.C.M. Van Riel, A.J.L. De Jong, and J.B.A. Van De Putte. 1997. Combination of sulphasalazine and methotrexate versus the single components in early rheumatoid arthritis: a randomized, controlled, double-blind, 52 week clinical trial. *Br. J. Rheumatol.* 36:1082.
15. Thomas R. and G.J. Carroll. 1993. Reduction of leukocyte and interleukin 1 concentrations in the synovial fluid of rheumatoid arthritis patients treated with methotrexate. *Arthritis Rheum.* 36:1244.
16. Snyder D.S. and E.R. Unanue. 1982. Corticosteroids inhibit murine macrophage Ia expression and interleukin-1 production. *J. Immunol.* 129:1803.
17. Schleimer R.P. 1990. Effects of glucocorticosteroids on inflammatory cells relevant to their therapeutic applications in asthma. *Am. Rev. Respir. Dis.* 141:559.
18. Ropes M.W., G.A. Bennett, S. Cobb, R. Jacox and R.A. Jansen. 1958. Revision of diagnostic criteria for rheumatoid arthritis. *Bull. Rheum. Dis.* 9:175.
19. Combe B., L. Edno, P. Lafforgue, C. Bologna, J.C. Bernard, P. Acquaviva, J. Sany and F. Bressolle. 1995. Total and free methotrexate pharmacokinetics, with and without piroxicam, in rheumatoid arthritis patients. *Br. J. Rheumatol.* 34:421.
20. Nowak O., M. Zieba, D. Zawinska, J. Rożniacki and M. Król. 1996. Changes of serum concentration of lipid peroxidation products in patients with pneumonia. *Monaldi Arch. Chest. Dis.* 51:188.
21. Crilly A., I.B. McInnes, A.G. McDonald, J. Watson, H.A. Capell and R. Madhok. 1995. Interleukin 6 (IL-6) and soluble IL-2 receptor levels in patients with rheumatoid arthritis treated with low dose oral methotrexate. *J. Rheumatol.* 22:224.
22. Danis V.A., G.M. Franic, D.A. Rathjen, D.M. Laurent and P.M. Brooks. 1992. Circulating cytokine levels in patients with rheumatoid arthritis: results of a double blind trial with sulphasalazine. *Ann. Rheum. Dis.* 51:946.
23. Seitz M., P. Loetscher, B. Dewald, H. Towbin, C. Rordorf, H. Gallati, M. Baggiolini and N.J. Gerber. 1995. Methotrexate action in rheumatoid arthritis: stimulation of cytokine inhibitor and inhibition of chemokine production by peripheral blood mononuclear cells. *Br. J. Rheumatol.* 34:602.
24. Chang D.M., M.E. Weinblatt and P.H. Shur. 1992. The effects of methotrexate on interleukin 1 in patients with rheumatoid arthritis. *J. Rheumatol.* 19:1678.
25. Elliott M.J. and R.N. Maini. 1995. Anti-cytokine therapy in rheumatoid arthritis. *Bailliere's Clin. Rheumatol.* 9:633.
26. Guerns P.A., B.L. Zuraw, J.H. Vaughan, D.A. Carson and M. Latz. 1989. Synovium as a source of interleukin 6 in vitro. *J. Clin. Invest.* 83:585.
27. Dinarello C.A. 1991. Interleukin-1 and interleukin-1 antagonism. *Blood* 77:1627.
28. Janero D.R. 1990. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. *Free Radical Biol. Med.* 9:515.
29. Sikora A. 1995. The chemiluminescence assessment of peripheral blood polymorphonuclear leukocytes metabolic activity in juvenile chronic arthritis. *Pol. J. Immunol.* 20:97.

C.M.
lation
divity
fluid.

small.

The material on this page was copied from the collection of the National Library of Medicine by a third party and may be protected by U.S. Copyright law.