Albumin-coupled methotrexate (MTX-HSA) is a new anti-arthritic drug which acts synergistically to MTX

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Objective. To evaluate the anti-arthritic effects of the new inflammation-targeted drug MTX-HSA and to investigate whether peripheral blood mononuclear cells (PBMC) are potential target cells for albumin-mediated drug delivery.

Methods. The murine model of collagen-induced arthritis (CIA) was used to measure the anti-arthritic effect of MTX, MTX-HSA or a combination of both ($n = 30$ to 35 per group). In addition, the uptake of fluorescence-labelled albumin (AFLc-HSA) in PBMC of 14 patients with RA was measured by fluorescence-activated cell sorting (FACS).

Results. In equivalent doses of 7.5 mg/kg intravenously (IV) twice a week, MTX-HSA is significantly ($P < 0.02$) superior to MTX in inhibiting the development of CIA and reducing the joint count as well as the number of affected paws. When given in lower doses as combination therapy, both drugs act synergistically ($P < 0.03$). A mean of 96, 72 and 64% of the CD14-, CD16- and CD20-positive cells from peripheral blood of rheumatoid arthritis (RA) patients showed an uptake of albumin after incubation with AFLc-HSA *in vitro*. This finding was not significantly different in comparison to healthy controls. In contrast, the number of CD3-positive cells taking up albumin is increased significantly in RA patients in comparison to controls ($26.3 \pm 12.9\%$ S.D. *vs* $11.6 \pm 7.3\%$ S.D.; $P = 0.005$).

Conclusion. The data show that the effectiveness of MTX-HSA in CIA is superior to MTX and that both drugs act synergistically. In addition, albumin appears to be taken up by peripheral blood cells, suggesting that they might be one of the potential target cells of this novel anti-arthritic treatment approach.

KEY WORDS: Methotrexate, Rheumatoid arthritis, Albumin, T lymphocytes, Macrophages.

A predominant feature of synovitis in rheumatoid arthritis (RA) is the development of a hypertrophic, oedematous and highly vascularized so-called pannus-like tissue that progressively invades and degrades adjacent cartilage and bone [1–4]. This tissue, which originates from the synovial lining layer of affected joints, consists of synovial fibroblasts (SFs), synovial macrophages and various infiltrating inflammatory cells such as activated T and B lymphocytes. The joint destruction is mediated by matrix-degrading enzymes released predominantly by activated SFs similar to mechanisms observed in malignant disease [5, 6]. Due to these activated properties of cellular components, both RA and various malignant diseases can be treated with antiproliferative drugs such as methotrexate (MTX) [7, 8], which has evolved into the most commonly used drug in the treatment of RA [9, 10].

On the other hand, at present progression of joint destruction cannot be completely inhibited by MTX treatment in most RA patients. One reason might be that large amounts of the administered MTX are eliminated by the kidneys within a short period of time, resulting in short plasma half-life of less than 24 h and a low drug concentration in the affected joints. Although increasing the dose of MTX frequently results in a higher therapeutic efficacy, it also leads to a higher risk of side-effects. These side-effects limit the maximum dose that can be adminis-

tered [9, 10]. The disadvantageous pharmacokinetic properties of MTX are therefore most likely to be the basis of numerous unsatisfactory treatment results [11–13].

To overcome the lack of specificity with regard to the target tissue and prolong the effective half-life, drugs can be covalently coupled to suitable drug carriers such as macromolecular proteins. These carriers should provide a high accumulation in the target tissue with a prolonged drug exposure time combined with low uptake rates in non-diseased tissue.

Recently we demonstrated that by covalent coupling of MTX to the plasma protein albumin (MTX-HSA), a drug conjugate is created that fulfils these requirements for enhanced and specific delivery of drugs into target tissue in malignant diseases as well as into synovial tissue in patients with RA: albumin conjugates were taken up intracellularly and were metabolized lysosomally by cells with a high protein and nitrogen demand such as tumour cells [14, 15] or SFs of RA patients [1–3, 5, 6, 16]. Furthermore, MTX-HSA has a plasma half-life of about 15 days, which is equivalent to HSA; it showed no immunogenicity and was well tolerated in a clinical Phase I trial for cancer treatment [17]. At present, the conjugate is under clinical investigation in Phase II clinical trials.

The potential use of albumin as a drug carrier in RA is favoured by the facts that the permeability of the blood–joint barrier for

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albumin in inflamed joints is markedly increased [18] and patients with active RA, similar to cachectic cancer patients [15], frequently develop hypoalbuminaemia due to increased albumin turnover, presumably caused by high consumption of albumin at sites of inflammation [19–21]. In accordance with this, we showed that in different animal models albumin conjugates accumulated in tumours as well as in inflamed joints of arthritic mice [16]. Moreover, MTX-HSA is superior to MTX in the suppression of tumour growth [14, 15] and development of arthritis [16] in rodent models. Moreover, in a human SCID mouse model of RA, in which synovial fibroblasts of patients with RA are co-transplanted with cartilage, we recently showed that the cartilage-invading synovial fibroblasts take up albumin conjugates intracellularly [16]. In this specific model, MTX and MTX-HSA both inhibited cartilage invasion and degradation with comparable efficiency [22].

Therefore, albumin appears to be a suitable drug carrier not only for tumour therapy but also for the treatment of RA, and the covalent coupling of MTX to albumin might increase therapeutic efficacy and reduce the side-effects of MTX.

However, several questions regarding the application of MTX-HSA in arthritis still need to be addressed: (1) do MTX and MTX-HSA act synergistically in the prevention of arthritis as they do in some experimental tumor models and (2) are SFs [16] the only cellular target of MTX-HSA, or might mononuclear cells be additional target cells for the treatment with albumin conjugates? In the work presented here, we therefore show the effects of the application of MTX-HSA alone or in combination with MTX in the murine collagen-induced arthritis (CIA) model, and we analyse the albumin uptake of peripheral blood mononuclear cells (PBMC) from RA patients *in vitro*.

Patients and methods

Reagents

Human serum albumin (HSA) was purchased from Pharma Dessau (Dessau, Germany). Diethylenetriaminepentaacetic acid (DTPA), dimethylsulphoxide (DMSO), *N,N'*-dicyclohexylcarbodiimide (DCC), and *N*-hydroxysuccinimide (HSI) were provided by Aldrich (Steinheim, Germany). For separation of the compounds, ultrafiltration units (exclusion size 30 kDa) from Millipore (Eschborn, Germany) were used. 5([4,6-Dichlorotriacin-2-yl]-amino)-fluorescein (AFLc), bovine type II collagen, anti-mouse-IgG Fc-specific biotin conjugate and methotrexate (MTX) were purchased from Sigma (Deisenhofen, Germany). Incomplete Freund's adjuvant and *Mycobacterium tuberculosis* H 37 RA were from Difco/Becton-Dickinson (Detroit, USA). Streptavidin–horseradish peroxidase (HRP) complex was obtained from Amersham Life Science (Freiburg, Germany). Ficoll was from Biochrom KG (Berlin, Germany). Anti-CD3 IgG1 kappa phycoerythrin (PE) conjugated, anti-CD14 IgG2a kappa PE conjugated, anti-CD16 IgG1 kappa PE conjugated and anti-CD20 IgG1 kappa PE conjugated were from DAKO (Hamburg, Germany).

Fluorescence labelling of human serum albumin with aminofluorescein (AFLc-HSA)

For the covalent coupling of AFLc to albumin in a 1:1 molar ratio, 4 g HSA (diluted in 20 ml saline), 20 ml 0.17 M NaHCO$_3$ and 15 ml methanol were combined. With continuous stirring 45 mg AFLc, diluted in 4 ml DMSO, was added. After 1 h, 350 ml H$_2$O was added, and the low-molecular-weight compounds were separated by ultrafiltration. This step was repeated five times. Finally, the purified AFLc-HSA conjugate was diluted in sodium bicarbonate buffer (0.17 M, pH 8.4), and the concentration of AFLc was determined by absorbance at 497 nm. Quality controls were carried out by HPLC analysis. No polymer formation was observed during the conjugation procedure. The HPLC profile of AFLc-HSA was consistent with the profile of HSA.

Coupling of methotrexate to human serum albumin (MTX-HSA)

MTX-HSA was prepared as described previously, using *N,N'*-dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (HSI) for activation and linking MTX to HSA [24].

Collagen-induced arthritis (CIA)

All animal experiments were approved by the Animal Welfare Committee according to the regulations of the German Federal Government. Two hundred and eighty-three male DBA/1 mice, 7–8 weeks of age, were obtained from M&B (Ry, Denmark). The mice were maintained in the central animal house at the University of Heidelberg. CIA was induced according to the procedure described by Wooley [25, 26]: Bovine type II collagen was dissolved in 0.1 M acetic acid at a concentration of 2 mg/ml by stirring for about 3 h at 4°C. To prepare the adjuvant, 3 mg heat-inactivated *Mycobacterium tuberculosis* was added to 1 ml incomplete Freund's adjuvant. Equal volumes of the collagen solution and the adjuvant were emulsified at 4°C. The mice were immunized by three intradermal injections of the emulsion at the base of the tail (50 μl each). Three weeks after the first immunization, the procedure was repeated. Within 2 weeks of the second immunization, about 70% of the mice developed arthritis characterized by erythema and swelling of the paws. In the majority of mice, the disease progressed to two or more paws. The severity of arthritis was graded on a score of 0–3 as described before [27, 28].

Anti-collagen II antibody ELISA

Serum was obtained from anaesthetized mice by intracardial puncture at the end of the treatment period, which was 2 weeks after the second immunization. The concentrations of antibodies against bovine collagen II in the serum of DBA/1 mice were measured by the enzyme-linked immunosorbent assay (ELISA) technique as described in [27]. Briefly, bovine collagen II was coated to 96-well microtitre plates (7 μg/well) by incubation for 24 h at 4°C. After washing with phosphate-buffered saline with 0.2 M NaCl and Tween 20 (PNT buffer) and incubation with 1% HSA in PNT buffer, the serum of CIA mice was added and incubated for 18 h at 4°C. As a standard, the serum of a CIA mouse with a known concentration of anti-collagen antibody (courtesy of A. M. Malfyt, Kennedy Institute of Rheumatology, London, UK) was added in different concentrations. After an other washing step 1 μg/ml biotinylated Fc-specific anti-mouse IgG was incubated for 2 h at room temperature. Following a washing step with PNP buffer the bound antibodies were detected by HRP-streptavidin complex and *o*-phenylenediamine (OPD) and the blue colour quantified at 450 nm in an ELISA-reader.

Treatment protocol in CIA

Toxicity studies were performed to evaluate the maximum tolerated dose (MTD): when the drugs were applied to five DBA/1 mice per group twice weekly, a MTD of 35 mg/kg body weight for MTX and 7.5 mg/kg for MTX-HSA was calculated.

The difference in the MTD between MTX and MTX-HSA is easily explained by the longer half-life of MTX-HSA which is about 2 days in comparison to <12 h for MTX, which results in a higher exposure of the mice to MTX-HSA in comparison to MTX when both drugs are given twice weekly.

To study the effect of MTX-HSA and MTX on the development of CIA, 267 DBA/1 mice were distributed randomly among seven groups. Two weeks after the first immunization with collagen (2 weeks before the onset of arthritis), treatment was started.

The animals were treated twice weekly for 4 weeks, each mouse receiving eight injections. One group received intraperitoneal injections of saline (group 1, control, $n = 63$). The mice in the six treatment groups ($n = 30$ to 36) were injected intraperitoneally with MTX either 7.5 or 35 mg/kg body weight, MTX-HSA either 1.6 or 7.5 mg/kg or both drugs in combination either 3.75 mg/kg MTX plus 0.8 mg/kg MTX-HSA or 17.5 mg/kg MTX plus 3.75 mg/kg MTX-HSA (Fig. 1). The dosage of the drugs was chosen either to obtain the maximal effect by using the maximum tolerated dose (see above) or to obtain the maximal difference between the single drugs in low concentrations and their combination in order to prove the synergy between both drugs. The calculated dose of

MTX-HSA was based on the amount of MTX bound to albumin. Therefore, mice which were treated with equivalent doses of either MTX-HSA or MTX received the same molar amount of the MTX component, but either covalently coupled or non-coupled to albumin. After 4 weeks of treatment, all mice were sacrificed according to the local animal welfare regulations, which constitute that mice must not be affected by arthritis for longer than 2 weeks. During the course of the experiment, the mice were inspected twice weekly for arthritis, side-effects of treatment, and were weighed. The percentages of mice affected by CIA in each animal group were calculated. Statistical significance was tested using the Fortran subroutine Fytest.
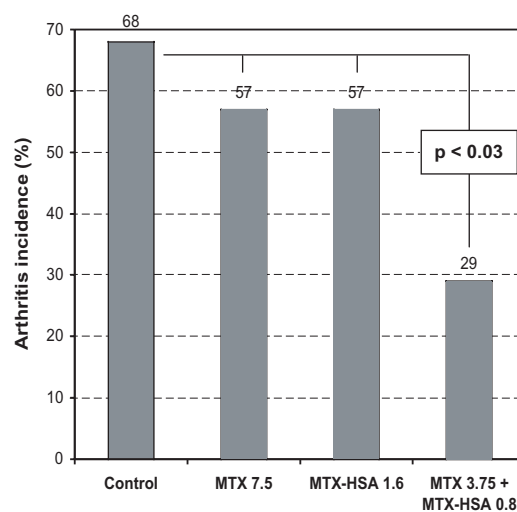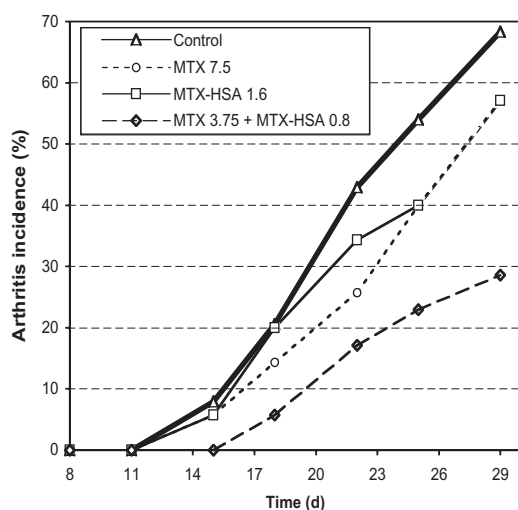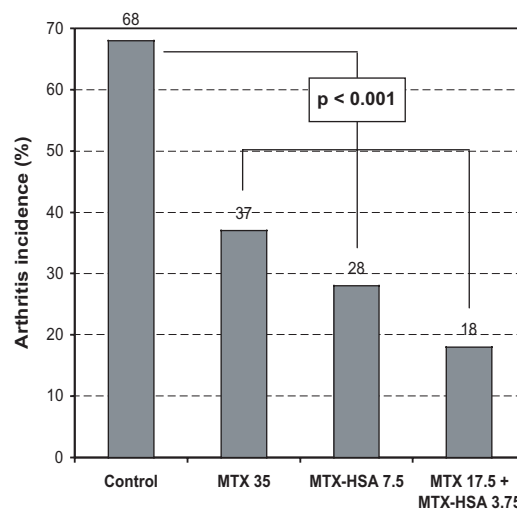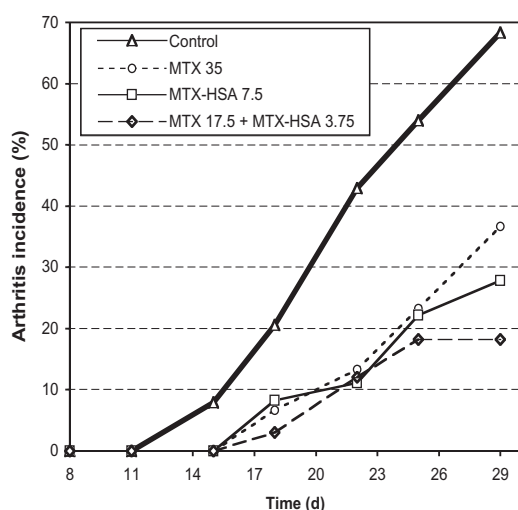

FIG. 1. Effect of different doses of MTX, MTX-HSA or a combination of both on the development of collagen-induced arthritis (CIA) of mice. The percentages of arthritic mice in each treatment group are presented (= arthritis incidence). Treatment was started 2 weeks before the onset of the disease. The animals were treated for 4 weeks and received two intraperitoneal injections weekly. The dosage of both drugs is calculated by the molecular amount of MTX which is given as a free drug (MTX) or covalently coupled to albumin (MTX-HSA). MTX and MTX-HSA suppressed significantly ($P < 0.001$) the development of arthritis (lower row). 7.5 mg/kg MTX-HSA (lower row) was significantly ($P < 0.02$) more effective in the suppression of the onset of arthritis than the equivalent dose of MTX (upper row). At least a 5-fold higher dose of MTX was required to achieve the levels of suppression seen with MTX-HSA. When both drugs were combined and given in 50% of the dose of the drugs alone, in the low-dose schedules (upper row) a significantly ($P < 0.03$) reduced incidence of arthritis in comparison with treatment with a single drug was found. This difference was also seen in higher dosages of the drugs (lower row), but here it did not reach statistical significance.

TABLE 1. Characteristics of patients and healthy controls

	RA patients	Healthy controls
n	14	11
Mean age (yr)	55.6 ± 16.7 (s.d.)	39.9 ± 11.6 (s.d.)
Female gender	8 (57%)	6 (55%)
Disease activity score (DAS28) [41]	3.2 ± 1.9 (s.d.), range 0.8–6.0	–
C-reactive protein (CRP) in plasma (mg/l)	10.1 ± 10.7 (s.d.), range 2.0–39.1	–
Years from disease onset	8.2 ± 3.1 (s.d.), range: 1.8–22.0	–
Treatment regimen	Pred. only, 3	–
	Pred. + MTX, 2	
	Pred. + MTX + LEF, 2	
	Pred. + MTX + SSZ, 1	
	Pred. + MTX + ETA, 4	
	Pred. + MTX + INF, 1	
	ETA, 1	

Abbreviations: Pred., prednisone or prednisolone; MTX, methotrexate; LEF, leflunomide; SSZ, sulphasalazine; ETA, etanercept; INF, infliximab.

Fluorescence signal of peripheral blood mononuclear cell populations after in vitro incubation with AFLc-HSA

Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll centrifugation of plasma from 14 RA patients and 11 healthy controls. All RA patients fulfilled the American College of Rheumatology (ACR; formerly the American Rheumatism Association) criteria for the diagnosis of RA [29] and written consent was obtained according to the Declaration of Helsinki. The study has been approved by the ethics committee of the University of Heidelberg.

The characteristics of patients and healthy controls are described in Table 1.

1×10^6 PBMC/ml in 24-well plates were incubated in the presence of 50 μg/ml AFLc-HSA at 37°C for 15–17 h. Afterwards the cells were vigorously washed three times and PE-labelled antibodies against CD3, CD14, CD16 and CD20 were added and incubated on ice for 20 min. After a further washing step the PBMC were analysed by fluorescence-activated cell sorting (FACS) (Becton-Dickinson, Detroit, USA) for the AFLc-HSA signal (FL1) and binding of the PE-labelled antibodies (FL2).

Results

Treatment results

Of the control animals, 68% developed arthritis during the course of the study.

When low doses of the drugs were used (Fig. 1, upper row), the incidence of arthritis in mice treated with 7.5 mg/kg MTX or 1.6 mg/kg MTX-HSA was 57%, which was not significantly different from the control group. However, when 50% of the doses of each drug are given in combination (3.75 mg/kg MTX plus 0.8 mg/kg MTX-HSA) a significantly ($P < 0.03$) lower incidence of arthritis of 29% can be observed. Therefore, both drugs act synergistically, as an only additive effect would result in the same incidence of arthritis as 7.5 mg/kg MTX or 1.6 mg/kg MTX-HSA alone.

When high doses of the drugs are given (Fig. 1, lower row) MTX-HSA as well as MTX or the combination of both drugs show a significantly better reduction of incidence of arthritis (37, 28 and 18% respectively) in comparison to the control group ($P < 0.001$ each). Therefore, although the synergistic effect of the combination of MTX-HSA and MTX in comparison to the single drugs is still detectable in this high-dose setting, it fails to reach significance.

Remarkably, 7.5 mg of MTX-HSA/kg was significantly better ($P < 0.02$) than equivalent doses of MTX in inducing reduction in the incidence of arthritis. Moreover, a high MTX-dose of 35 mg

MTX/kg was required to obtain a reduction of incidence of arthritis to 37%, which is closest to the incidence of arthritis of 28% obtained with 7.5 mg MTX-HSA/kg.

Similar results were found when instead of incidence of arthritis, the arthritis score or the number of affected paws of all mice were analysed (Fig. 2). In the group of mice which received lower doses of the drugs (7.5 mg/kg MTX, 1.6 mg/kg MTX-HSA or 3.75 mg/kg MTX plus 0.8 mg/kg MTX-HSA) an effect of the combination of both drugs in the reduction of the arthritis score (control 3.0 ± 0.4 s.e.m. and 2.2 ± 0.5, 2.7 ± 0.6 and 1.0 ± 0.3 respectively in the treatment groups) and on the reduction of the number of affected paws (control: 1.2 ± 0.1 s.e.m. and 0.9 ± 0.2, 1.2 ± 0.2 and 0.5 ± 0.1 respectively in the treatment groups) could be shown, which was statistically significant ($P < 0.04$ and $P < 0.05$ respectively). In contrast, the arthritis scores and the number of affected paws were significantly ($P < 0.005$ and $P < 0.007$ respectively) reduced by the treatment with high doses of both drugs or the combination of both (35 mg/kg MTX, 7.5 mg/kg MTX-HSA and 17.5 mg/kg MTX plus 3.75 mg/kg MTX-HSA) but no significant synergistic effect of the combination of both drugs was detectable. Remarkably, when only the mice which developed arthritis were analysed no significant difference was found between the treatment groups, showing that once the mice developed arthritis the treatment did not alter the course of the disease.

Anti-collagen type II antibody concentrations in serum

While no anti-collagen type II antibodies were detectable in the serum of naïve DBA/1 mice, in almost all mice that received immunization with intradermal collagen type II for the induction of CIA, anti-collagen type II antibodies were detectable in the serum in a range from 20 up to 1000 μg/ml (Fig. 3) at the end of the treatment period. The concentrations of anti-collagen type II antibodies were significantly reduced by MTX, MTX-HSA or a combination of both, when high doses of the drugs are used ($P < 0.05$; Fig. 3, left lower row) and a synergistic effect between MTX-HSA and MTX could be observed when lower concentrations of the drugs were used (Fig. 3, left upper row). Significantly higher concentrations of collagen type II antibodies could be found in the mice which developed arthritis in comparison to the mice not showing signs of arthritis (Fig. 3, right column).

FACS analysis of PBMC

PBMC from 14 patients with RA and 11 healthy individuals were incubated with fluorescence-coupled albumin (AFLc-HSA) and afterwards AFLc-HSA uptake (FL1) in different cell populations
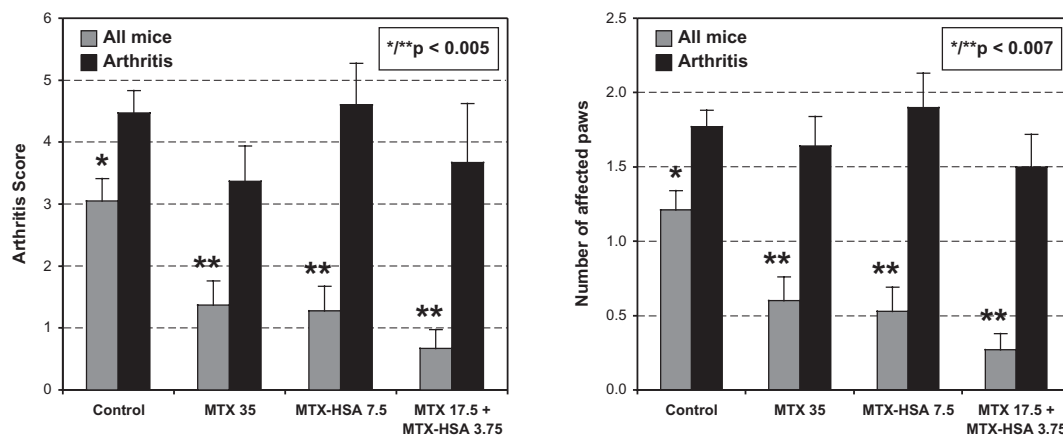
Low dose
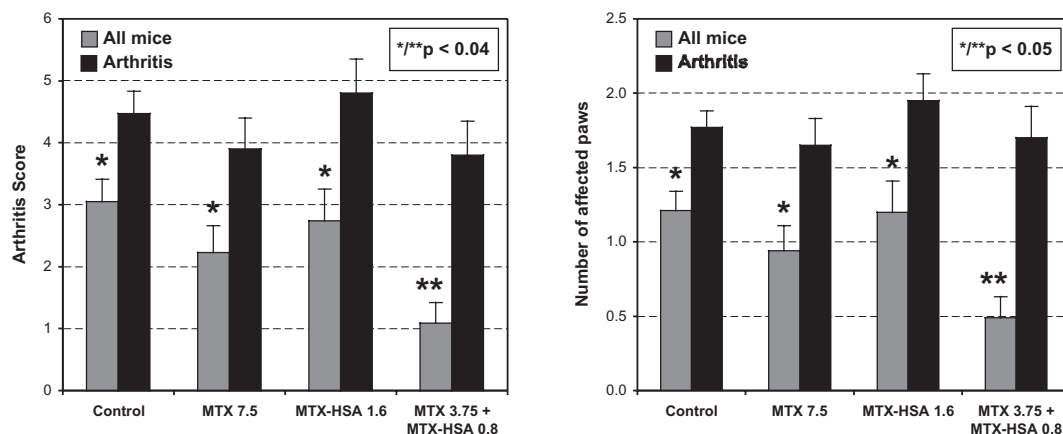
High dose

FIG. 2. Arthritis scores and number of affected paws of mice with CIA treated with different concentrations of MTX, MTX-HSA or both. The arthritis score is calculated by a clinical score of 0–3 per paw (maximum score of 12 per mouse). In parallel to the effects of the treatment on arthritis incidence (Fig. 1), MTX, MTX-HSA and a combination of both significantly reduces the arthritis score and the number of affected paws when given in high doses (lower row, $P < 0.007$). Moreover, a significant synergistic effect of MTX and MTX-HSA on arthritis score ($P < 0.04$) and the number of affected paws ($P < 0.05$) is detectable when the drugs are given in lower doses (upper row). However, when only the mice which developed arthritis were analysed, no significant difference was found, which shows that the treatment did not influence the severity of arthritis in mice once they did develop arthritis. Results are shown as mean $\pm$ S.E.M.

was analysed by FACS (Fig. 4). The absolute cell counts in the peripheral blood of RA patients were 3.8/nl for CD3-positive cells, 1.4/nl for CD14-positive cells, 1.6/nl for CD16-positive cells and 1.6/nl for CD20-positive cells. Almost all CD14-positive cells showed FL1 positivity after incubation with AFLc-HSA *in vitro*, regardless of whether they were derived from patients with RA ($96.1 \pm 8.2\%$ S.D.) or from healthy individuals ($98.7 \pm 1.8\%$ S.D.). In the CD16-positive cell population, the percentage of cells with FL1 positivity was $72.1 \pm 21.7\%$ S.D. (RA) and $62.0 \pm 15.3\%$ S.D. (controls) and in the CD20-positive cell population $64.7 \pm 31.3\%$ S.D. and 53.4 ± 20.5 S.D. respectively. Although the rate of FL1-positive cells in the CD16- and CD20-positive populations was higher in RA patients, this difference failed to reach statistical significance.

In contrast to the CD14-, CD16- and CD20-positive cell populations, in the CD3-positive population the rate of cells showing a fluorescence signal after incubation with fluorescence-labelled albumin was much lower. Only $26.3 \pm 12.9\%$ S.D. of the CD3-positive cells from patients with RA showed a fluorescence signal after incubation with AFLc-HSA. However, in comparison with

the healthy individuals, which have a rate of $11.7 \pm 7.2\%$ S.D., this is significantly increased ($P = 0.005$). When we correlated the results with clinical and laboratory data from the patients we could not find a significant correlation between AFLc-HSA fluorescence in any of the cell populations and the parameters sex, age, C-reactive protein (CRP) concentration in the serum, disease activity score (DAS28), time since first diagnosis of RA or the actual medication. Moreover, the frequency of fluorescence-positive cells after AFLc-HSA incubation was not correlated with the absolute count of the different cell populations in the peripheral blood.

Discussion

Albumin is the largest transportable source of nitrogen and energy in the body and can be used to carry drugs to cells with a high nitrogen and energy demand such as the cells involved in the synovial inflammation of RA [14–16, 30]. We have recently shown that MTX-HSA is more effective than equivalent concentrations of
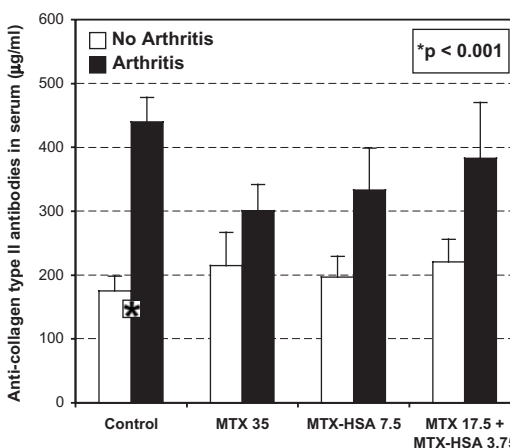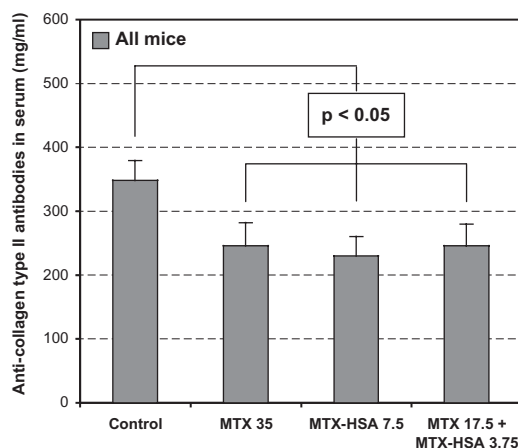
Low dose
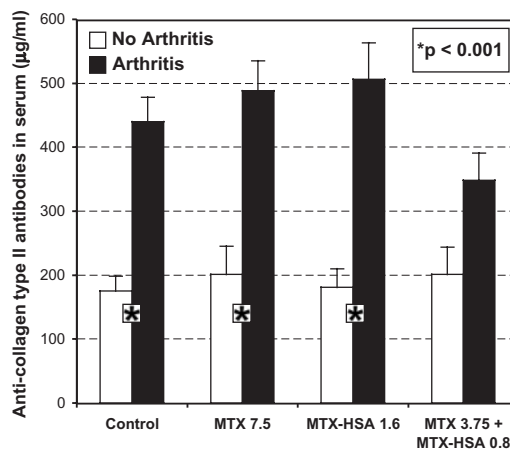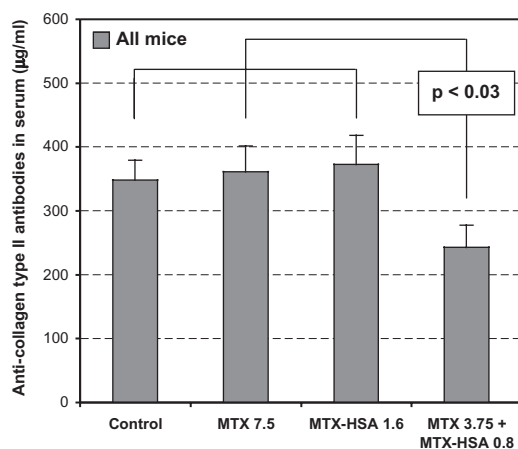
High dose

FIG. 3. Anti-collagen type II-antibodies in the serum of CIA mice treated with different doses of MTX-HSA, MTX or a combination of both as determined by the ELISA-technique. The serum was sampled at the end of the treatment period. In parallel to the effects of the treatment on arthritis incidence (Fig. 1), arthritis scores and the number of affected paws (Fig. 2) MTX, MTX-HSA and a combination of both significantly ($P < 0.05$) reduces the concentrations of anti-collagen type II antibodies when given in high doses (left bottom). A synergistic effect ($P < 0.03$) between both drugs is detectable when given in lower doses (left top). Mice which developed arthritis had significantly higher concentrations of antibodies in comparison to mice which did not develop arthritis. This was found regardless of whether the mice received MTX, MTX-HSA or a combination of both drugs in low doses (right top, $P < 0.001$) or high doses (right bottom, $P < 0.001$). Results are shown as mean ± S.E.M.

MTX in preventing CIA [16]. Remarkably, in these experiments a high MTX dose of 35 mg of MTX/kg twice weekly was required to obtain a result comparable to 7.5 mg of MTX-HSA/kg, showing the superior effect of MTX-HSA in comparison to MTX.

In the data reported here we now show that when both drugs were combined, a synergistic effect was observed. Small doses of both drugs in combination were sufficient to obtain effects which resembled those which could be obtained when high doses of the single drugs were applied. This result corresponds to the observation that in RA the combination of MTX with other disease-modifying drugs (DMARDs) or cytokine inhibitors, which have different cellular targets and modulate different pathophysiological pathways, leads to synergistic effects in terms of increased therapeutic efficacy. In clinical settings this has been shown for the combination of MTX with cyclosporin A [31], sulphasalazine and hydroxychloroquine [32] and leflunomide [33] as well as the combination of MTX with inhibitors of tumour necrosis factor-α (TNF-α) [34, 35]. Although MTX-HSA most likely shares the majority of modes of action with MTX, the two agents need to be

regarded as pharmacologically separate drugs as they have different distributions in the body [16] and might therefore target different cell populations. Moreover, while MTX is taken up intracellularly by folate receptors [36, 37], albumin conjugates are internalized into cells by endocytosis [38]. These different mechanisms of cellular uptake might therefore result in a different pattern of cells, which are susceptible to treatment with either MTX-HSA or MTX. Therefore, our findings that MTX-HSA and MTX have synergistic effects in the prevention of CIA might be due to the fact that MTX-HSA and MTX complement each other in their anti-inflammatory effect by targeting different cell populations. The significance of the synergistic effect of MTX and MTX-HSA is supported by the fact that we have previously found similar synergistic effects between MTX-HSA and MTX in the growth retardation of Walker-256 carcinosarcoma in rats [23].

In human RA, the therapeutic effect of MTX can usually be observed after a treatment period of at least 4 weeks. As a similar effect can be expected in murine CIA, we started the treatment of the mice with MTX, MTX-HSA or both before the onset of
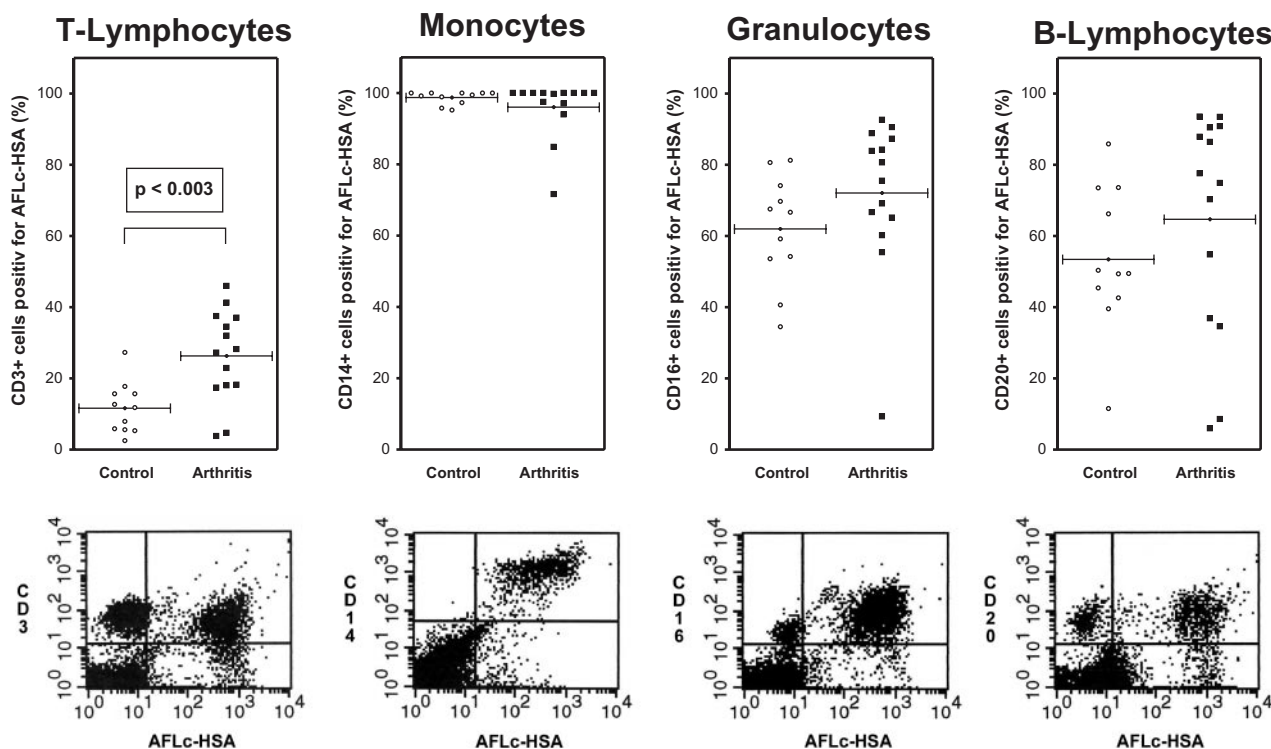
FIG. 4. Percentage of positive fluorescence signal of subpopulations of PBMC from patients with RA and healthy controls after incubation with 50 µg/ml fluorescence-labelled albumin (AFLc-HSA) *in vitro*. As the cells were vigorously washed after incubation, the fluorescence signal most likely represents intracellular uptake of the albumin by the cells. Monocytes, and to a lesser degree granulocytes and B lymphocytes, show the highest percentage of albumin uptake *in vitro*. Although granulocytes and B lymphocytes from RA patients showed a higher rate of albumin uptake in comparison to the control group, no statistical difference was reached. In contrast, T lymphocytes had a generally lower rate of albumin uptake, but this was significantly ($P < 0.003$) increased in cells derived from patients with RA. In the lower row representative pictures of the FACS analysis of different populations of PBMC after incubation with AFLc-HSA are shown. Vertical bars represent the mean values.

arthritis to evaluate the effect of the treatment on the course of arthritis in the first weeks after onset, which in CIA mice is the most active period of arthritis. Moreover, local animal welfare regulations did not permit the follow-up of CIA mice for longer than 2 weeks after the onset of arthritis. Therefore, we focused on the effect of the treatment on the incidence of the disease rather than the reduction of established arthritis which would have required a much longer and ethically non-permissible follow-up period of the arthritic mice.

Murine albumin can be obtained in small quantities only, which makes it difficult to perform *in vivo* experiments. Therefore, in the present study we used MTX-coupled human albumin (HSA) to treat DBA/1 mice. As HSA is xenologous in mice, it has the potential to be immunogenic. However, we have previously shown that the distribution of rat and human albumin in rats is not different. Moreover, in rats or nude mice, multiple injections of various HSA–drug conjugates were well tolerated and the animals did not show signs of allergy or anaphylaxis [14, 15, 39]. Therefore, as albumin is highly conserved in the evolution of mammals and as rats and mice as rodents have many similarities, we hypothesize that interspecies differences between mice and rats in the distribution of human albumin can be neglected. Moreover, as HSA is coupled to the immunosuppressive drug MTX (MTX-HSA), the immunogenicity might be further reduced.

Little is known about the uptake of MTX by inflammatory cells in human RA. However, it has been shown that polygluta-mated MTX is found in leucocytes of patients with RA [40]. Knowledge of the cellular distribution of MTX-HSA in RA would give more insight in the mechanisms of its effect and the reason for the synergistic effect with MTX described above.

In previous studies we identified synovial fibroblasts [16] as potential target cells for albumin-mediated drug delivery in arthritis. For this cell population, which is found in an activated state in synovitis and which is involved in the key pathophysiological processes of RA, we could show by confocal laser scanning microscope that albumin is taken up intracellularly and that it is metabolized in the lysosomal compartment of the cell. In the work presented here, we chose to use the FACS technique to study the uptake of fluorescence-labelled albumin in cell populations of the peripheral blood of patients with RA and of healthy individuals. In contrast to confocal microscopy, the FACS technique provides information about the frequency of cells which take up albumin and, by double-staining with PE-labelled antibodies specific for cell surface markers, it provides information about the albumin uptake of different cell types without a previous separation step. However, although the cells were vigorously washed to remove albumin, FACS staining, in contrast to confocal microscopy, does not completely exclude binding of albumin to the cell surface.

Using double-staining, monocytes and granulocytes in particular, but also B lymphocytes, stained by anti-CD14, anti-CD16 and anti-CD20 antibodies respectively showed a positive fluorescence signal after incubation with AFLc-HSA (Fig. 4) indicating the intracellular uptake of albumin. However, there was no significant difference in the frequency of fluorescence-positive cells between patients with RA and the control group, although there was a trend towards a higher rate in granulocytes and B lymphocytes from RA patients in comparison with the control group. In T lymphocytes stained by anti-CD3 antibodies the frequency of cells with positive fluorescence as a surrogate marker

of albumin uptake was generally lower in comparison to the other cell types (Fig. 4). However, in the T lymphocytes the frequency of cells which showed fluorescence after incubation with AFLc-HSA was significantly increased in cells from patients with RA in comparison with the control group.

We therefore conclude that a number of blood-borne effector cell populations of synovial inflammation such as monocytes, granulocytes and B lymphocytes can take up albumin to a high rate and therefore are targeted by a treatment with albumin–drug conjugates. T lymphocytes comprise an additional cell population which is attributed to have an important role in RA. It has been shown that in peripheral blood and the synovial tissue of patients with RA, T lymphocytes are found which have an activated phenotype [41]. Our observation that T lymphocytes from the peripheral blood of patients with RA take up albumin in a significantly higher frequency than those of healthy control individuals shows that the albumin demand of this, most likely activated T lymphocytes, is increased in RA. It therefore represents an interesting target cell population for albumin–drug conjugates. Further studies have to show whether the uptake of MTX-HSA by PBMC is different from that of MTX and whether this might be a cause of the synergism between the both drugs.

In conclusion, MTX-HSA acts synergistically with MTX to prevent murine CIA. Therefore, the option of a combination therapy of both drugs should be included in further clinical studies with MTX-HSA in RA. MTX-HSA most likely exerts its effects through accumulation of the drug in effector cell populations of RA. In addition to synovial fibroblasts, which we previously identified as target cells for albumin-mediated drug delivery in RA, monocytes, granulocytes and T and B lymphocytes, which all were shown to play a significant role in the pathophysiology of this disease, might be further targets of this novel treatment approach. In the work presented here we did not evaluate the effects of MTX-HSA on synovial inflammation, cartilage damage or bone erosions. However, in a recent work we found an inhibitory effect of both MTX and MTX-HSA on synovial fibroblast invasion and destruction of cartilage in a human SCID mouse model of RA [22]. Nevertheless, the potential differential effects of MTX and MTX-HSA on joint inflammation and destruction remain to be determined in further studies.

Rheumatology	Key messages
	• Albumin-coupled MTX (MTX-HSA) prevents CIA and is superior to MTX in equivalent concentrations. • MTX and MTX-HSA act synergistically when given in combination. • Albumin–drug conjugates are taken up by peripheral blood mononuclear cells from patients with RA.

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