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Flare-up of antigen-induced arthritis in mice after challenge with oral antigen

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SUMMARY

Mice with unilateral chronic mBSA-induced arthritis were orally challenged with mBSA. Three hours after antigen challenge clear flare-up of the chronic arthritis was demonstrable as detected by an increase in the ^{99m}Tc uptake of the knee joint and the reaction continued for at least 2 days. The contralateral non-arthritic knee joint was not affected. The dose of mBSA needed to induce a flare-up in nearly all mice within a group was in the order of 20 mg. After oral challenge with 10 or 5 mg of mBSA the incidence was lower and flare-up reactions were only rarely observed after challenge with 2·5 or 1·25 mg mBSA. Histology of knee joints taken at 24 h after oral challenge of 20 mg mBSA revealed an increase in the number of cells in the infiltrate in the synovial tissue and exudate in the joint space, the most conspicuous sign being the increase of PMN. Passage of macromolecules through the gastrointestinal mucosa may be an important principle in the perpetuation of human chronic arthritis.

Keywords antigen-induced arthritis flare-up reactions oral administration of antigen protein absorption gut

INTRODUCTION

Flare-up of chronic joint inflammation has been described after i.v. antigen injection in mice with ongoing antigen-induced arthritis (van de Putte *et al.*, 1983). An important principle is leakage of antigen from the circulation into the chronically inflamed synovial tissue in amounts sufficient to induce a flare-up; the first feature of the reaction, i.e. granulocytes in the synovial tissue, was already observed at 2 h after antigen administration (Lens, 1984). Studies on the mechanism involved in the flare-up phenomenon have revealed that the arthritic knee joint behaves as a hyper-reactive area due to retention of immunoreactive cells in the chronically inflamed synovial tissue (Lens, van den Berg & van de Putte, 1984a). Further studies have shown that in particular the local retention of specific T cells is of prime importance (Lens *et al.*, 1984b).

The above mentioned experiments indicate that antigen in the circulation can induce an exacerbation of ongoing smouldering arthritis and may contribute to the perpetuation of inflammation. This may be relevant to the pathogenesis of chronic arthritis if non-invasive conditions could be found in which sufficient amounts of exogenous antigen enter the circulation. Obviously under physiological conditions the greatest amount of exogenous antigen is usually presented to the body in the gut, making this a likely site for uptake of antigen into the circulation. Previous studies have shown that macromolecules can enter the circulation in an immunoreactive

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form after oral injection (André *et al.*, 1979; Pang, Walker & Block, 1981; Roberts *et al.*, 1981; Stokes, Swarbrick & Soothill, 1983).

In the present study we have investigated whether a flare-up of antigen-induced arthritis can be induced by oral administration of the antigen. Five weeks after the induction of arthritis with methylated bovine serum albumin (mBSA) mice received mBSA by intubation into the stomach. The fate of antigen in the circulation and in the knee joints was followed using radiolabelled antigen (^{125}I-mBSA). Our data support the view that antigenic material can pass the intestinal mucosa in amounts sufficient to induce an exacerbation of ongoing chronic arthritis.

MATERIALS AND METHODS

Animals. Male C57Bl mice, aged 7–9 weeks and weighing 24–26 g at the start of the immunization, were used.

Iodination of antigen. ^{125}Iodine labelling of mBSA was performed by the chloramine-T method (Hunter & Greenwood, 1962). ^{125}I-mBSA was separated from free ^{125}I by sephadex G25 fractionation.

Arthritis induction. Animals were immunized with mBSA in Freund's complete adjuvant as previously described (Brackertz, Mitchell & MacKay, 1977) using *Bordetella pertussis* organisms (National Institute of Public Health, Bilthoven, The Netherlands) as an additional adjuvant. On day 21 arthritis was induced by intra-articular (i.a.) injection of 60 μg mBSA in 6 μl saline into the right knee joint; 6 μl saline was injected into the left joint as control.

Oral antigen administration. In the chronic phase of the joint inflammation, 5 weeks after the initial induction, the animals received after an overnight fast mBSA in 0·5 ml water by intubation into the stomach. The amount of mBSA administered varied from 1·25 to 60 mg. Control animals received 0·5 ml water by intubation. In order to study the kinetic of mBSA in the blood and in the joint after oral challenge, mice received 20 mg ^{125}I-labelled mBSA (25 μCI = 0·93 MBq) by intubation into the stomach (see below). Two days before challenge potassium iodide (50 μg/ml) was added to the drinking water to prevent accumulation of free ^{125}I into the thyroid gland.

Measurement of joint inflammation. Arthritis was quantitated by the ^{99m}Tc-pertechnetate (^{99m}Tc) method as previously described in detail (Kruijsen *et al.*, 1981; Lens *et al.*, 1984c). Briefly, 10 μCi (0·37 MBq) ^{99m}Tc was injected s.c. and the uptake in the knee joints was measured 40 min later by external gamma-counting. Mean values were calculated from three consecutive measurements with a duration of 20 s, alternating the right and the left knee. The severity of the inflammation was expressed as the ratio between the mean uptake in the right knee joint and that in the left (R/L ratio). Ratio's correlate well with histological inflammation scores (Lens *et al.*, 1984b). An increase in the R/L ratio of 15% or more after challenge with antigen was taken to indicate a flare-up of arthritis.

Histology. At 24 h after oral administration of 20 mg mBSA mice were killed by ether anaesthesia. Both knee joints were removed in toto and fixed in 4% phosphate-buffered formalin. After decalcification of the joints in 5% formic acid, the tissues were processed and embedded in paraffin wax. Whole joint sections (7 μm) were prepared and stained with haematoxylin & eosin.

Measurements of mBSA in blood. At various hours after oral administration of 20 mg ^{125}I-mBSA (25 μCi) mice were sedated by ether anaesthesia and blood samples taken by cardiac puncture. Samples were collected in 3·8% sodium citrate solution to prevent clotting. The amount of radioactivity in the total blood volume was measured by gamma-counting of 10 μl samples, and expressed as the % of the administered dose at time zero, taking 1/15 part of the body weight as total blood volume (Wish, Furth & Storey, 1950). Blood samples were centrifugated, plasma collected and stored at -20°C until use. Free ^{125}I in the plasma was separated from ^{125}I bound to high molecular weight material by Sephadex G25 fractionation. In Table 2 this fraction is referred as ^{125}I-protein. The amount of ^{125}I-mBSA in this protein fraction was investigated by co-precipitation with anti-mBSA serum, produced in rabbit according to standard procedure (Hudson & Hay, 1980). Anti-mBSA serum (100 μl) was added to 10 μl of column ^{125}I protein fraction made up to an optimal mBSA concentration for precipitation (1 mg/ml). After incubation for 1 h at 37°C and 1 h at 4°C the fraction was spun down (30 min, 2,500***g***, 4°C). The amount of precipitated radiolabel never exceeded the amount of radiolabel precipitating non-specifically (10%) with the used anti-mBSA serum. This indicates that the amount of ^{125}I-mBSA is at most 10% of the ^{125}I-protein.

Radioactivity measurements in the knee joint. At various hours after oral challenge of 20 mg ^{125}I-mBSA (25 μCi) mice were killed by ether anaesthesia and the knee joints removed *in toto*. Bone marrow was removed from the extremities by extensive flushing and released ^{125}I was removed from the joints by repeated washing with saline after fixation in 4% formalin. The amount of radioactivity associated with the joint tissue after this procedure was measured in a gamma-counter.

RESULTS

Occurrence of flare-up reaction after oral antigen administration

Groups of mice with unilateral mBSA-induced chronic joint inflammation were orally challenged with different doses of mBSA ranging from 1·25 to 60 mg. The severity of the arthritis was measured by ^{99m}Tc uptake of the joints and expressed as a ratio of the uptake in the right arthritic knee *vs* that in the left non-arthritic knee (R/L ratio). An increase of 15% in this ratio was taken to indicate a flare-up. Table 1 shows the incidence of flare-up of the chronic joint inflammation in the different groups measured 24 h after challenge. High incidence of flare-up was found after oral administration of 60 and 20 mg mBSA. The number of responders was lower in groups of mice challenged with 10 or 5 mg mBSA and flare-up reactions were rarely observed after challenge with 2·5 or 1·25 mg mBSA. The intensity of the flare-up reactions was not significantly different between the responders in the groups of mice challenged with either 60, 20 and 10 mg mBSA (Table 1). For comparison one group of mice was challenged i.v. with 300 μg mBSA. In eight out of nine mice flare-up reactions were observed and the intensity was slightly higher ($P=0·05$) as compared with flare-up reactions seen after oral mBSA challenge (Table 1).

Time course of flare-up of arthritis

A dose of 20 mg mBSA was chosen to study the time course of the flare-up reaction since a dose of 60 mg mBSA caused variable side effects in the intestine whereas a dose of 10 mg mBSA did not consistently induce a flare-up reaction (Table 1). A flare-up was already demonstrable 3 h after antigen administration, continued for at least 2 days and decreased in 4 days to values of ^{99m}Tc

Table 1. Flare-up of joint inflammation, dependent on the dose of orally administered mBSA

Dose of mBSA (mg)	Proportion of animals showing flare-up*	Δ ^{99m}Tc uptake (%)† of the responders
60	5/6	21 ± 4
20	8/9	29 ± 8§
10	11/16	28 ± 7
5	3/11	23
2·5	1/5	21
1·25	1/12	27
0·3‡	8/9	40 ± 12

* Arthritis was measured before and after oral challenge by ^{99m}Tc uptake and expressed as R/L ratio (see Materials and Methods). A fifteen per cent increase in the R/L ratio was taken to indicate a flare-up.

† Mean ± s.d.

‡ Injected i.v.

§ Significant difference between oral and i.v. antigen challenge (Student's t-test, two tailed, $P=0·05$).

uptake as measured in a control group of mice which received water instead of mBSA (Fig. 1). Morphological studies, carried out on whole knee joint sections of mice killed 24 h after oral challenge with mBSA, showed characteristics of an acute type of inflammation superposed on the chronic inflammation already present i.e. an increase in the number of cells, predominantly PMN, in the infiltrate in the synovial membrane and in the exudate in the joint space. These features are similar with those seen in flare-up reactions after i.v. antigen challenge (van de Putte *et al.*, 1983). Morphological investigation of the contralateral non-arthritic joints revealed no sign of inflammation after oral mBSA challenge.

Measurements of radioactivity in blood and joints

The amounts of radiolabel in the blood of immune and non-immune mice were measured at various times after oral challenge with 20 mg ^{125}I-mBSA ($\pm$25 μCi). The total radioactivity measured in the blood of immune and non-immune mice was similar (Table 2). At 30 min after challenge approximately 5% of the orally administered dose of radioactivity was found in the blood. The level slightly increased hereafter and a peak value of 8·8% was found between 1 and 3 h after challenge. Table 2 further shows that only a small portion of the radiolabel measured in the blood was bound to high molecular weight material (indicated as ^{125}I-protein). Co-precipitation with anti-mBSA serum did not yield detectable amounts of ^{125}I-mBSA in the ^{125}I-protein fraction indicating that less than 10% represents immunoreactive antigen. Table 3 shows the amounts of radioactivity accumulated in the arthritic and non-arthritic joints of immune mice and in normal joints of non-immune mice. The knee joints were removed *in toto*, bone marrow was flushed from the extremities and free ^{125}I was removed from the joints by repeated washing after formalin fixation. More radiolabel was found in arthritic joints compared with non-inflamed joints, at all hours after challenge. At 1 h the R/L ratio of the measurements in the right arthritic knee joint and the left non-arthritic knee joint was 1·7 and increased to 3·8 at 7 h. Table 3 further shows that the amounts of radioactivity in non-inflamed joints of immune and non-immune mice were comparable.

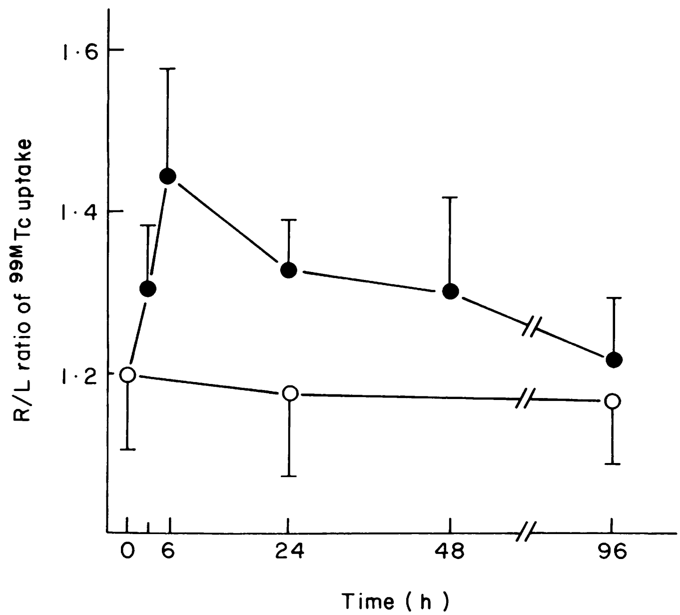

Fig. 1. Time course of flare-up of mBSA-induced arthritis measured after oral challenge of 20 mg mBSA (●). Severity of inflammation is expressed as R/L ratio of ^{99m}Tc uptake (mean+s.d., $n=5$). ○ represent measurements (mean−s.d.) of chronic arthritis 0, 24 and 96 h after oral challenge of water ($n=20$, 4 and 6, respectively). Significantly increased values were measured at 3, 6, 24 and 48 h after challenge of mBSA (Student's t-test, two tailed, $P<0·02$).

Table 2. Measurements of radioactivity in blood of immune and non-immune mice after oral challenge with 20 mg ^{125}I-mBSA

Hours after challenge	Radioactivity in total blood (%)*		^{125}I-protein† (%) of total ^{125}I in plasma		Amount‡ (μg) of ^{125}I-protein in total blood	
	Immune	Non-immune	Immune	Non-immune	Immune	Non-immune
0·5	5·2§	5·0				
1	7·6	7·4	0·06	0·16	0·9	2·4
1·5	8·3	7·0				
2	8·8	8·0	0·14	0·42	2·5	6·7
3	8·5	7·5				
6	4·0	4·3	0·05	0·26	0·4	2·2
24	0·1	0·1	2	3	0·4	0·6

Values represent mean of three mice.

* Expressed as % administered dose. Blood volume was calculated: 1/15 of body weight (Wish *et al.*, 1950).

† ^{125}I bound to protein in plasma was separated from unbound ^{125}I by sephadex G25 fractionation. The radioactivity bound to protein is expressed as a % of the total radioactivity in the plasma.

‡ Calculated from the percentages and the administered dose.

§ Blood was collected by cardiac puncture.

Table 3. Measurements of radioactivity in knee joint of immune mice and non-immune mice after oral antigen challenge*

Hours after challenge	Immune mice			Knee joints of non-immune mice
	Arthritic joint (R)	Non-inflamed joint (L)	R/L	
1	1,867(1,625–2,225)	1,083(803–1,344)	1·7(1·5–2·2)	1,041(766–1,241)
3	1,915(1,775–2,159)	976(804–1,251)	2·0(1·4–2·5)	812(506–1,012)
7	3,075(2,251–4,415)	808(614–1,064)	3·8(2·7–4·5)	763(497–1,082)
24	1,177(1,115–1,251)	505(431–572)	2·4(2·3–2·5)	512(378–676)

Values represent the mean of at least three mice (range indicated between brackets).

* Mice were challenged with 20 mg ^{125}I-mBSA (±25 μCi).

DISCUSSION

Oral mBSA administration in mice with chronic low grade mBSA induced joint inflammation resulted in a flare-up of the arthritis without affecting the contralateral non-arthritic knee joint. The orally induced flare-up reaction was demonstrable already 3 h after antigen challenge and had a duration of more than 2 days (Fig. 1). Histological investigation showed an increase in the number of cells, predominantly PMN, in the infiltrate in the synovial membrane and in the exudate in the joint space. High oral doses of mBSA (10 mg or more) were needed to induce to phenomenon, probably due to the passage of only small amounts of immunoreactive mBSA from the gastrointestinal mucosa into the circulation.

Local hyper-reactivity in the chronically inflamed joint has been shown to play an important role in the flare-up reaction (Lens *et al.*, 1984). The local hyper-reactivity is probably mediated by immunoreactive cells present in the chronically inflamed synovial tissue like T lymphocytes and plasma cells (Lens *et al.*, 1984d). T lymphocytes seem of prime importance, since the flare-up reaction is suppressed by pre-treatment with anti-lymphocyte serum while a local Arthus reaction seems less important since the reaction is not influenced by pre-treatment with cobra venom factor (Lens *et al.*, 1984b).

In addition to local hyper-reactivity of the chronically inflamed joint an important principle in the induction of the flare-up is the leakage of antigen from the circulation into the chronically inflamed tissue. Previous experiments made clear that an i.a. dose of as little as 10 ng mBSA is sufficient to induce a flare-up in a hyper-reactive joint and this amount is apparently reached after a single i.v. injection of 10 μg mBSA, being the lowest i.v. dose able to induce a significant flare-up (Lens *et al.*, 1984a). After oral challenge most of the antigen is digested and the peak amount of immunoreactive antigen entering the circulation is at most 10% of the ^{125}I-protein and therefore anyhow below 250 ng (Table 2). Nevertheless, a rather continuous supply of these small amounts over several hours is apparently sufficient to induce a flare-up in the chronically inflamed joint.

Comparison of the flare-up reactions induced after oral or i.v. antigen challenge (Lens *et al.*, 1984a) revealed that the reaction is less pronounced and of shorter duration after oral challenge. Moreover, the accumulation of radiolabelled protein in the right (R) arthritic joint *vs* that in the left (L) contralateral non-inflamed joint was more pronounced after oral challenge, resulting in a high R/L ratio at 7 h after challenge. This higher ratio after oral as compared with i.v. challenge may be related to the fact that increased antigen entrance into the arthritic joint probably occurs at the onset of acute inflammation (flare-up), 2–3 h after antigen challenge, at the moment of granulocyte-mediated increase in vascular permeability (Wedmore & Williams, 1981). By that time most of the radiolabelled protein has already been cleared from the circulation after a single i.v. injection (Lens, 1984), in contrast to the high level of circulating ^{125}I-protein after oral antigen supply.

After oral administration of foreign proteins most of the material is digested in the stomach. The use of radiolabel as a marker for intestinal uptake of proteins may lead to overestimation of the absorption, since labelled fragments of the digested original protein may bind to high molecular weight serum constituents (Udall *et al.*, 1981), thereby mimicking the uptake of macromolecules. Trichloroacetic acid insoluble radioactivity in the blood, which is used by some authors as a measure for antigen absorption (André *et al.*, 1979), may therefore largely represent unrelated protein. Our data are in support of the latter since simple immunoprecipitation of the radiolabelled protein after oral challenge with ^{125}I-mBSA did not reveal detectable amounts of immunoreactive antigen, indicating that less than 10% (the lower limit of the assay) of the ^{125}I-protein represents mBSA. These low amounts are in accordance with the small amounts of immunoreactive antigen found in the blood by radioimmunoassay after oral intake of unlabelled protein (Swarbrick, Stokes & Soothill, 1979). Another striking feature of antigen uptake after oral ingestion is the large variability. Intestinal absorption may differ considerably between mice of one strain and among different strains (Stokes *et al.*, 1983) and this is probably related to variance in IgA levels in the gastrointestinal tract (André *et al.*, 1979). Our observations on the flare-up after oral challenge with various antigen doses (Table 1) may also point to considerable variations in antigen absorption since we found responders and non-responders within one dose group whereas a more uniform reaction pattern was observed after i.v. injection with all mice within a given dose group being either responders or non-responders (unpublished data).

The polyarthritic nature of the rheumatoid disease suggests a propagation of the joint inflammation by an inflammatory stimulus in the circulation, the gut being a likely site due to its daily antigenic load. Under physiological circumstances in the gut only small amounts of macromolecules pass unchanged from the gastrointestinal mucosa into the blood and this may only have pathogenic consequences for the joint, when it already possesses local hyper-reactivity. On the other hand, under pathological circumstances in the gut, e.g. inflammation due to infections or allergic reactions, enhanced uptake of macromolecules has been observed (Bloch et al., 1979; Kilshaw & Slade, 1980; Roberts *et al.*, 1981) and this may have pathogenic consequences in terms of induction of arthritis in normal joints. Evidence for the involvement of material from the gut in the induction of human arthritis has emerged from the development of arthritis in some patients who have undergone intestinal bypass surgery (Stein *et al.*, 1981) and in patients suffering from infections in the gut (Bennett, 1978; Berden, Muytjens & Van de Putte, 1979). The role of food and food allergy in the pathogenesis of human arthritis is yet unknown (Moment, 1980; Ziff, 1983), but enhanced uptake of macromolecules has been observed during food allergic reactions in the gastrointestinal tract (Dannaeus *et al.*, 1979). A few case studies have reported exacerbations of joint inflammation after ingestion of specific foods like milk and cheese (Parke & Hughes, 1981; Williams, 1981; Little, Steward & Fenessy, 1983). In addition, a higher incidence of autoimmunity, including rheumatoid arthritis, was scored in patients with IgA deficiency (Cunningham-Rundles *et al.*, 1981) which probably possess a decreased intestinal barrier. Our present data show that oral antigen can induce an exacarbation of smouldering arthritis and indicate the possibility that the gastrointestinal tract may be involved in the pathogenesis of some forms of human arthritis.

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