The gut as an inductive site for synovial and extra-articular immune responses in rheumatoid arthritis

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Abstract

Objectives—To analyse the immunological interactions between the gut lymphoid tissue, synovium, and peripheral blood compartments in patients with rheumatoid arthritis (RA) and ankylosing spondylitis (AS).

Methods—Patients with RA and AS and healthy controls were orally or parenterally immunised with an influenza virus vaccine. Antigen-specific antibody responses were measured at the single cell level by ELISPOT assay using lymphocytes isolated from peripheral blood and from enzymatically dispersed synovial tissues.

Results—Both oral and parenteral immunisations induced antigen-specific antibody-secreting cells in the synovial tissue of patients with RA. Parenterally immunised patients with RA showed significantly decreased antigen-specific antibody responses in peripheral blood compared with patients with AS and with healthy controls. In contrast, oral vaccination evoked comparable peripheral blood antibody responses in all three study groups.

Conclusions—Despite a decreased immune responsiveness in the systemic compartment, the functional status of the gut-associated lymphoid tissue in patients with RA is intact. In addition, there is evidence that the lymphocytes in the inflamed joints are accessible for signals both from the systemic and mucosal compartments. The findings of immunological 'cross-talk' are relevant to future vaccination and tolerisation procedures in patients with RA.

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occur.^{6–7} Changes of gastrointestinal permeability,^{8–9} altered pattern of bacterial colonisation^{10–11} as well as beneficial effects of fasting have been reported in patients with RA.¹² Furthermore, fasting has been shown to ameliorate the disease activity and simultaneously decrease the bowel permeability in patients with RA.¹³ Direct evidence for the role of the gastrointestinal tract in the immunoregulation of experimental arthritis has been demonstrated by the tolerogenic effects of an oral administration of type II collagen in an experimental rat model with RA.¹⁴

Despite the evidence that the immune system of the gastrointestinal tract may modulate experimental arthritis, the gut-associated lymphoid system has not been carefully studied in rheumatic patients. We therefore set up a model system for evaluation of the gut-associated lymphoid system as an inductive site for an antigen-specific immune response. This is performed by oral immunisation with killed influenza virus vaccine and subsequent assessment of peripheral blood B-lymphocyte responses at the single cell level. In addition, synovial tissue lymphocytes obtained from patients with RA, were analysed regarding antigen-specific antibody production in response to the mucosal influenza vaccine challenge.

There are conflicting data concerning the systemic B-cell responsiveness in patients with RA, both at the cellular and humoral level. Hypo- and hyperreactivity as well as intact immune responses have been reported.^{15–18} To analyse the systemic B-cell responsiveness at the single cell level rheumatic patients were immunised parenterally with influenza vaccine.

Methods

SUBJECTS AND VACCINE

The table provides a summary of experimental procedures and clinical features of patients included in this study. We believe that all participants in this study have been previously naturally exposed to the influenza virus. Thus we consider that all the B-cell responses to influenza vaccine seen are of secondary nature. In the first set of experiments 25 patients with RA, 9 patients with AS and 19 healthy volunteers were orally immunised with 5 ml of killed and highly purified influenza virus vaccine (Merieux Flu Vaccine, Lyon, France, kindly donated by Dr A Rosetzky, Rhone-Poulenc, Institute Merieux), dissolved in tap

Summary of experimental procedures and clinical features of patients included in the study

Type of immunisation	Diagnosis (experiment no in brackets)	Source of lymphocytes	No of subjects	Sex (M/F)	Age mean (SD) years	Disease duration mean (SD) years	RF-pos (%)	Treatment			
								NSAID (%)	Corticosteroids (%)	DMARD (%)	Cytotoxic drugs (%)
Oral	RA (1)	blood	25	7/18	56 (12)	15 (12)	76	68	28	56	20
	RA (3)	synovium	10	2/8	58 (10)	20 (12)	70	90	30	50	10
	AS (1)	blood	9	8/1	48 (10)	24 (13)	—	100	—	11	—
	Controls (1)	blood	19	8/11	36 (8)	—	—	—	—	—	—
Parenteral	RA (2)	blood	14	4/10	58 (15)	14 (9)	100	71	43	36	43
	RA (3)	synovium	9	4/5	53 (17)	16 (10)	100	67	56	33	44
	AS (2)	blood	9	6/3	42 (11)	15 (16)	—	100	—	44	11
	Controls (2)	blood	10	3/7	40 (12)	—	—	—	—	—	—
Non-immunised	RA (3)	synovium	12	0/12	61 (13)	12 (6)	75	67	8	42	17

water containing 1 g of bicarbonate. In the second set of experiments 14 patients with RA, 9 patients with AS and 10 healthy controls were parenterally immunised with 0.5 ml of the above vaccine, according to the manufacturer's recommendations. In the third set of experiments 10 of the orally and 9 of the parenterally immunised patients with RA were subjected to synovectomy seven days after the immunisation with influenza virus vaccine. A control group of 12 women with RA was not immunised before the synovectomy. The following pharmacological treatment was employed:

(A) NSAID (non-steroid anti-inflammatory drugs): indomethacin, Sulindac, Diclofenac, Naproxen, Ibuprofen

(B) Corticosteroids: prednisolone <10 mg/day

(C) Disease modifying anti-rheumatic drugs (DMARD): sulphasalazin, Natrium-aurothiomalas, Auranofin

(D) Cytotoxic drugs: podophyllotoxinum, methotrexate, ciclosporin

The medical history of three patients who had not been immunised in the third set of experiments was not available.

This study was approved by the Ethical Committee of the University of Göteborg.

ISOLATION OF LYMPHOCYTES FROM PERIPHERAL BLOOD AND SYNOVIUM

Blood was collected immediately before vaccination and seven days later, since B-cell responses at the single cell level peak at day 7 after systemic immunisation.¹⁹ Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on a Ficoll-Hypaque (Sigma Chemicals, St Louis, MO, USA) density gradient, washed and resuspended in Iscoves medium (Gibco, Glasgow) supplemented with 5% fetal calf serum (FCS) (Gibco). The PBMC were used immediately after isolation for the enzyme-linked immunospot assay (ELISPOT) as previously described.^{20, 21}

Synovial tissue samples were obtained from patients with RA in conjunction with synovectomy. Single cell suspensions from these tissues were prepared by enzymatic digestion with Dispase (Boehringer-Mannheim, Germany) as previously described.²² The isolated lymphocytes were washed, resuspended in Iscoves medium supplemented with 5% FCS and immediately used for the ELISPOT assay.

REAGENTS

Affinity-purified F(ab')₂ fragments of goat anti-human IgG (Cappel Laboratories, PA, USA), goat anti-human IgA and goat anti-human IgM (Jackson Laboratories, PA, USA) were used as coating reagents in the ELISPOT assay²⁰ at a concentration of 5 µg/ml. Influenza vaccine was diluted in phosphate buffered saline (PBS) 1:20 and used as coating reagent in the ELISPOT assay. The coating concentrations were previously optimised by checkerboard-titration. Affinity purified, biotinylated F(ab')₂ fragments of goat anti-human IgG, IgA, and IgM antibodies (Tago, Burlingame, CA) were used at the developing stage. Avidin-alkaline phosphatase (ALP) was purchased from Sigma.

ELISPOT ASSAY FOR ENUMERATION OF IMMUNOGLOBULIN AND SPECIFIC ANTIBODY SECRETING CELLS

The ELISPOT assay was used to determine the numbers of immunoglobulin secreting cells of a given isotype and of antigen-specific antibody secreting cells. The principle of this assay has been described elsewhere.²³ Briefly, a single cell suspension of lymphocytes is incubated on a solid phase coated with a relevant antigen, followed by an immunoenzymatic procedure which will allow the visualisation of antigen-specific B-cells as singular dark spots. Wells in the lids of 24 well culture cluster plates (Costar, Cambridge, MA) were coated (see 'reagents') overnight at 4°C. The plates were then washed with tap water and blocked with 5% PBS-FCS for one hour. One hundred µl of mononuclear cell suspension was added into each coated well and incubated for 3.5 hours at 37°C. After another wash, biotinylated goat anti-human IgA, IgG and IgM antibodies, diluted 1:750 in PBS containing 0.05% Tween 20 were applied followed by incubation with 0.5 µg/ml of avidin-ALP in PBS-Tween. After addition of the phosphatase substrate solution (5-bromo-4-chloro-3-indolyl phosphate) (Sigma) spots were enumerated under low magnification. Each spot represents one spot forming cell (SFC). All the assays were done in duplicate and at cell concentrations of 1×10^4 , 1×10^5 , 1×10^6 , and 1×10^7 /ml.

STATISTICAL ANALYSIS

Statistical analysis for differences between groups was carried out by the Mann-Whitney test.

Results

To assess the functional properties of the gut-associated lymphoid tissue, patients with RA, AS and healthy controls were orally immunised with an influenza virus vaccine and the subsequent immune response was recorded in the blood at the single cell level. No side effects of the oral immunisation were observed in any subject. To exclude variations in the kinetic pattern of the immune responsiveness to the antigen between patients with RA, AS and controls, PBMC from one subject in each group were analysed at days 0, 4, 7 and 10. The kinetics of the antigen specific antibody production was very similar for these three subjects: no influenza-specific SFCs at day 0, a few at day 4, a peak response at day 7, and a decreasing number of SFCs at day 10 (fig 1).

ANTIGEN-SPECIFIC B-CELL RESPONSES TO ORAL IMMUNISATION IN PATIENTS WITH RA AND AS

In the first set of experiments PBMC of the orally immunised participants were analysed before and seven days after the vaccination regarding the frequency of antigen-specific SFC. Before the immunisation no antigen specific SFCs were detected. Seven days later antigen specific SFCs were found in the majority of the orally immunised subjects. All three groups showed a similar frequency and isotypic distribution of the antigen-specific SFC (fig 2A). There were considerable interindividual variations for the B-cell responses within all the groups. The immune response was defined as positive if more than five antigen-specific SFC/10⁶ PBMC were detected seven days after the immunisation.²⁴ The frequency of responders was 15/25 (60%) for the patients with RA, 7/9 (78%) for the patients with AS and 14/19 (74%) for the control group. There was no significant difference with respect to the magnitude of the antigen-specific B-cell response in patients with RA treated with cytotoxic drugs compared with other forms of pharmacotherapy.

ANTIGEN-SPECIFIC B-CELL RESPONSES TO PARENTERAL IMMUNISATION IN PATIENTS WITH RA AND AS

In the second set of experiments the impact of a parenteral vaccination with influenza vaccine on the frequency of antigen-specific SFC was

analysed in peripheral blood. Before the immunisation antigen specific SFCs were not detected. Seven days later antigen specific SFCs were found in all but one (a patient with RA) subjects. This antigen-specific B-cell response to the influenza vaccine was 10–50 fold higher than that recorded in orally immunised subjects (fig 2B). The number of influenza-specific SFCs of IgG-class after parenteral immunisation was significantly lower in patients with RA compared with controls ($p < 0.01$) and patients with AS ($p < 0.05$). A similar, although not statistically significant trend was seen for IgA-specific B-cell responses. In contrast, the antigen-specific IgM responses were similar in all groups. There was no significant difference for the magnitude of the antigen-specific B-cell response in patients with RA treated with cytotoxic drugs compared with other forms of pharmacotherapy.

Figure 2 Numbers of anti-influenza SFC in peripheral blood seven days after immunisation in (A) orally immunised subjects and (B) parenterally immunised subjects. Anti-influenza SFC were not detected before the immunisation. Results are expressed as geometric means (SEM) of SFC per 10⁶ PBMC.

Figure 1 Numbers of anti-influenza SFC in peripheral blood before and on days 4, 7 and 10 after oral immunisation in a patient with RA, AS and a healthy volunteer, respectively.

ACCESSIBILITY OF SYNOVIAL TISSUE FOR IMMUNOLOGICAL SIGNALS FROM SYSTEMIC AND MUCOSAL COMPARTMENTS IN PATIENTS WITH RA

In the third set of experiments the accessibility of lymphocytes from the mucosal and systemic compartments to the articular compartment was evaluated. Synovial tissue samples were obtained from patients with RA, who seven days earlier had been orally or parenterally immunised with influenza virus vaccine. Both oral and parenteral immunisations induced an antigen-specific B-cell response in synovial tissue (fig 3). This response was significantly higher compared with patients with RA who had not been immunised (fig 3). The frequency of responders in each group was 6/10 for the orally immunised and 7/9 for the parenterally immunised. In contrast, only in two of the 12 non-immunised patients could a few antigen-specific antibody producing cells be detected (fig 3). The magnitude of the B-cell response was on average four fold higher in parenterally immunised subjects compared with that seen in orally immunised subjects. Notably, in one orally immunised patient we found antigen-specific SFC in the synovium but not in the peripheral blood. To analyse the propensity of influenza antigen committed

B-cells to home from blood to synovium after oral versus parenteral immunisation we have calculated the proportions of antigen-specific B-cells in synovial and blood compartments. Our results show that the relationship between geometric means of the numbers of antigen-specific synovial B-cells and corresponding peripheral blood B-cells is 0.32, 0.32, and 0.67 for IgG, IgA, and IgM isotypes in the orally immunised patients. In contrast, corresponding values in parenterally immunised patients were 0.07, 0.05, and 0.12.

The total number of immunoglobulin-producing cells in the synovial tissue was of the same magnitude in all three groups studied (fig 4).

Discussion

Although RA is an immunologically mediated disease and the mucosal immune system is the major lymphoid compartment of the human body there is scant information available on the immunological status of this area in rheumatic diseases. The results of this study suggest that the gut-associated lymphoid system is immunologically intact in patients with RA and AS. In addition, our data indicate that the joint-associated lymphoid tissue is accessible for immunological signals from both the systemic and mucosal compartments.

Mucosal administration of an antigen will induce a differentiation of antigen committed B-cells. These cells will enter the peripheral circulation and subsequently home to different mucosal tissues.^{25 26} Our results demonstrate that the mucosal deposition of the influenza virus vaccine induced a systemic antigen-specific response in both the rheumatic patients and the healthy controls. The magnitude, kinetics as well as the isotypic pattern of the B-cell response in peripheral blood were very similar between the groups. In this respect it has recently been proved that mucosal B-cell responses after mucosal antigen exposure closely correlate to the frequencies of antibody secreting cells in peripheral blood.^{27 28} Thus our results suggest that there is no alteration in mucosal immune responsiveness in patients with RA. The mucosa-associated lymphoid tissue in the gut of lupic MRL lpr/lpr mice also displays intact functional properties²⁹ despite a severe systemic immune anergy.³⁰

All patients included in this study received treatment, sometimes with drugs displaying immunomodulating properties. It has recently been reported that administration of NSAIDs may affect the permeability of the gut.³¹ These putative changes of permeability did not, however, affect the systemically recorded immune response to a mucosally presented antigen, since the groups of patients with RA and AS all on NSAID-treatment, responded equally well to the control group. This indicates that the gut-associated lymphoid system is not significantly altered by NSAIDs.

Fourteen of the 25 orally immunised patients with RA were treated with disease modifying anti-rheumatic drugs (DMARD),

Figure 3 Numbers of anti-influenza SFC in synovial tissue samples obtained from patients with RA seven days after oral and parenteral immunisation. Synovial tissue lymphocytes from non-immunised patients with RA were used as controls. Results are expressed as geometric means (SEM) of SFC per 10⁶ synovial mononuclear cells (SMC). P-values (* $p < 0.05$, ** $p < 0.01$) refer to comparisons between controls and immunised groups.

Figure 4 Numbers of total immunoglobulin SFC in synovial tissue samples seven days after immunisation in 10 orally, nine parenterally and 12 non-immunised patients (= controls) with RA. Results are expressed as geometric means (SEM) of SFC per 10⁶ SMC.

compounds known to have a potent systemic immunomodulating effect. However, neither treatment with DMARD nor cytotoxic drugs reduced the mucosal immune responsiveness to vaccination. In contrast, an impaired B-cell response after parenteral immunisation was commonly observed in the patients with RA, irrespective of the type of antirheumatic treatment. This finding agrees with Whaley *et al.*¹⁸ but contrasts with the results of Devey *et al.*, and Pelton *et al.*^{15, 16} These seemingly conflicting data may be due to the severity of RA, a factor of importance when assessing the status of the immune responsiveness. Also differences in choice and administration of immunogen as well as methodological aspects of the analysis of the immune response could influence the results. Indeed, the serological analysis may not be quantitative enough to permit the detection of subtle differences. In this respect, the results obtained at cellular level by the ELISPOT assay have the advantage of giving a precise and sensitive estimate of the frequency of antigen-specific B-cells at a defined moment and in a defined body compartment.

Immunological interactions between the systemic, mucosal and articular compartments might provide insight into the pathogenesis of rheumatic diseases. This study shows the presence of antigen-specific B-lymphocytes in the synovial tissue, both after oral and parenteral immunisation. The synovial influenza-specific B-cell responses after systemic immunisation has been previously reported by Pelton *et al.*¹⁶ The magnitude of the synovial B-cell response in our study is comparable to that seen in peripheral blood after oral immunisation. This is the first study to demonstrate an immunological connectivity between the gut and the synovial tissue in patients with RA. There are at least three potential pathways to induce a synovial B-cell reactivity in response to mucosal immunisation. Firstly, the mucosally deposited influenza antigen could have entered the circulation and subsequently the synovium, and once there activated synovial antigen-specific resting B-cells. Carefully performed studies, however, have shown that after a massive oral antigen intake (grams) only minute amounts (nanograms) of antigenic material pass the intestinal barrier.³² We believe therefore that of the minute amount of influenza virus antigen (130 µg) deposited in the gastrointestinal tract of patients participating in our study only traces (subpicogram quantities) would enter into the joint, and would be unable to evoke a synovial B-cell response. Secondly, intestinal antigen-specific B-cells could have been triggered in situ by the oral vaccination and then migrated to the synovium. Jalkanen *et al.* have demonstrated that migration of lymphocytes to the synovial tissue is controlled by specific adhesion molecules present on high endothelial venules.³³ Our results indicate that circulating antigen-specific B-cells triggered by oral exposure display a strong propensity to home to synovium compared with circulating B-cells

triggered by parenteral immunisation. Thirdly, the oral immunisation might have activated antigen-specific mucosal T-cells. After migration and homing to the synovial tissue these T-cells could interact with antigen-committed B-cells to induce an antibody production. Our preliminary data indicate that the cell-surface marker HML1, restricted to intra-epithelial T-lymphocytes in the gut,³⁴ is also found on some synovial lymphocytes. A site-directed traffic of lymphocytes from the gut to the articular compartment would raise the possibility of oral vaccination against, for example, septic arthritis, and of oral tolerance induction to collagen type II or proteoglycans.

We have recently shown that human synovial tissue exerts strong antigen presenting properties.²¹ Our present results, together with our previous report favour bilateral communication between articular and systemic lymphoid compartments. These findings are important since an intact function of the gut-associated lymphoid tissue and an immunological connection between extra-articular and synovial compartments are prerequisites for future vaccination and toleration studies in RA. Recent results^{35, 36} suggest that oral toleration may be a valuable treatment of certain autoimmune diseases.

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