Cytokines in experimental autoimmune vasculitis:
Evidence for a Th2 type response

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Abstract

Objective
To investigate the pathogenic role of cytokines in the development of experimental autoimmune vasculitis.

Methods
BALB/c mice were immunized with human IgG-ANCA from a patient with WG. Control mice were immunized with normal human IgG. Levels of mouse IgG-ANCA and other autoantibodies were determined. The mice lungs and kidneys were examined for the development of vasculitis. Levels of interleukin-1β (IL-1β), IL-2, IL-4, IL-6, interferon-γ (IFN-γ) and TNFα were determined by ELISA two weeks after immunization of the mice.

Results
Mice immunized with human IgG-ANCA developed anti-human IgG-ANCA (Ab2) and anti-anti-human IgG-ANCA (mouse IgG-ANCA = Ab3), while the controls did not develop these antibodies. The mice that were immunized with human IgG-ANCA developed perivascular mononuclear cell infiltrates in the lungs, suggesting vasculitis. Levels of IL-4, IL-6 and TNFα but not IL-1β, IL-2 and IFN-γ were significantly elevated in the mice 2 weeks after immunization with IgG-ANCA.

Conclusion
Our results suggest a pathogenic role for IL-4, IL-6 and TNFα in the initiation phase of autoimmune vasculitis. This suggests that a Th2 type immune response is responsible for the initiation of experimental autoimmune lung vasculitis, similar to Wegener’s granulomatosis in humans.

Key words
Vasculitis, autoimmunity, cytokines, interleukin.
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Introduction

Anti-neutrophil cytoplasmic antibodies (ANCA) are autoantibodies directed against proteins within the cytoplasmic granules of neutrophils and monocytes. ANCA have been classified into two types based on the fluorescent staining pattern of ethanol-fixed human neutrophils: cIgG-ANCA which stain cytoplasmic granules with a diffuse granular pattern, and pIgG-ANCA which stain the perinuclear area of neutrophils (1). cIgG-ANCA react predominantly with proteinase-3 (PR3), a 29 kD serine protease of the neutrophil azurophil granules, and are highly specific for Wegener’s granulomatosis (WG) (2).

We have recently described a new model of lung vasculitis induced by immunizing mice with a pathogenic human IgG-enriched ANCA (3, 4). In this model experimental vasculitis is induced in mice by immunizing them with human IgG-enriched ANCA (IgG-ANCA) derived from a patient with Wegener’s granulomatosis. Following immunization the mice developed high levels of anti-human IgG-ANCA (anti-idiotype antibodies = Ab2) and three months later they developed anti-anti-human IgG-ANCA (anti-anti-idiotype antibodies = mouse IgG-ANCA = Ab3). The generation of mouse IgG-ANCA was associated with inflammatory infiltrates around the small blood vessels in the lungs, simulating in these mice the early pulmonary pathologic lesions seen in patients with Wegener’s granulomatosis (3, 4).

The pathogenesis of vasculitis is complex and involves complicated mechanisms leading to the necrotizing inflammation of blood vessels. Recently it became evident that inflammatory cytokines and adhesion molecules may be important in the pathogenesis of vasculitides (4-6). The aim of the present study was to evaluate the importance of cytokines in the initiation of the vasculitis in our mouse model, and to examine whether it is a Th1, Th2, or a mixed type immune response. Our results point to an important role for inflammatory Th2 type cytokines in the initiation of experimental autoimmune vasculitis.

Materials and methods

Mice

Female BALB/c mice were purchased from Tel-Aviv University. The mice were 14 weeks old at the initiation of the experiments.

Purification of human IgG-ANCA

Human IgG-ANCA were purified from the plasma obtained by plasmapheresis of a patient with active WG. The plasma was loaded onto a goat anti-human IgG CNBr-activated Sepharose column (Sigma, St. Louis, MO). IgG was eluted from the column with SM MgCl2, and the eluate was dialyzed against phosphate buffered saline (PBS). The IgG gave a cIgG-ANCA pattern by immunofluorescence, and was reactive with the α-fraction (isolated azurophil granules which contain PR3) but not with myeloperoxidase (MPO) (data not shown, see refs. 3, 7).

Immunization protocol

Twenty female BALB/c mice were immunized intra-dermally in the hind footpads with the purified human IgG-ANCA (10 µg/mouse) in complete Freund’s adjuvant, and were boosted 3 weeks later with the same IgG-ANCA in PBS without adjuvant (10 µg/mouse). Twenty control mice were immunized with normal human IgG using the same protocol. In previous studies we have found that immunizing with normal human IgG in CFA is comparable to immunizing with CFA alone and therefore we used normal human IgG in CFA as the only control immunization protocol (data not shown). The mice were bled at 4 to 8 week intervals.

Isolation of α-fraction of neutrophils

The α-fraction of human neutrophils, containing PR3, was prepared according to a method previously described (8).

Indirect immunofluorescence for human and mouse IgG-ANCA

Human neutrophils were separated on a Ficoll-Hypaque gradient, smeared on glass slides, and ethanol fixed. The slides were incubated for 30 min. at room temperature with the test mouse or human sera diluted 1:20. After washing with PBS, FITC-conjugated anti-human or mouse IgG (Sigma), diluted 1:50, was
added and the slides were incubated for
30 min. The slides were washed and ex-
amined with a fluorescent microscope.

**ELISA for human and mouse anti-α-
fraction antibodies (Ab1, Ab3)**

Ninety-six well ELISA plates (Nunc,
Roskilde, Denmark) were coated with
the α-fraction (1 µg/well, diluted in
NaHCO3 0.05 M, 0.02% NaN3, pH 9.5)
overnight at room temperature. The
plates were washed with PBS/0.05%
Tween 20, and were blocked for 2 hours
with PBS/0.05% Tween/1% BSA
(Sigma). After washing, the sera diluted
1:200 in PBS/0.05% Tween/1% BSA,
were added and the plates were incubated
for 2 hours at room temperature. They
were washed again, and alkaline phos-
phatase-conjugated anti-human or anti-
mouse IgG (Jackson Laboratories, West
Grove, PA) was added for 1 hour. The
reaction was developed using p-
nitrophenyl phosphate as substrate, and
absorbance was read at 405 nm.

**Evaluation of cytokine levels**

Serum levels of murine cytokines (IL-
1β, IL-2, IL-4, IL-6, IFNγ and TNFα)
were measured by a solid phase ELISA.
ELISA kits were purchased from En-
dogen Inc. (Cambridge, MA), and from
Genzyme Diagnostics (Cambridge, MA).
The assay employs the quantita-
tive “sandwich” enzyme immunoassay
 technique in which a monoclonal anti-
body specific for the interleukin mol-
ecule has been pre-coated onto the poly-
syrene microtiter plate. Standards and
samples are introduced into the wells and
the interleukin present is bound by the
immobilized antibody. After washing
away any unbound proteins, the second
enzyme-linked polyclonal or mono-
clonal antibody specific for the interleukin
is added to the wells to “sandwich” the
interleukin immobilized during the first
incubation. Following a wash to remove
any unbound antibody-enzyme reagent,
a substrate solution is added to the wells
and color develops in proportion to the
amount of interleukin bound in the ini-
tial step. The color development is stop-
ped and the intensity of the color is mea-
ured. A curve is prepared, plotting the
optical density of the samples to this stan-
dard curve, the concentration of the interleukin in un-
known samples is then determined.

**Histology.** Mice were sacrificed at 8
months after the boost and the lungs and
kidneys were studied histologically fol-
lowing hematoxylin-eosin staining.

**Results**

**Autoantibody production in mice
injected with human IgG-ANCA**

Beginning at 6 weeks after immuniza-
tion, mice which had received human
IgG enriched ANCA developed detect-
able levels of mouse IgG-ANCA (Ab3)
as determined by and ELISA using the
α–fraction of human neutrophils (which
contains PR3, Fig. 1) and indirect im-
munofluorescence (Fig. 2). Significant
levels of mouse IgG-ANCA were de-
tected at 2 months after immunization
and the levels of mouse anti-α fraction
antibodies reached a peak at 3 months
after immunization, and then slightly
declined until the mice were sacrificed
(Fig. 1).

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**Fig. 1.** Levels of IgG-ANCA in mice immunized with hIgG-ANCA as determined by ELISA. Plates
were coated with α-fraction of neutrophils which contains PR3. The levels reached a peak at 3 months
and then slightly declined. The titer of anti-α fraction antibodies is represented by O.D. at 405 nm.

**Fig. 2.** Indirect immunofluorescence demonstrating binding of mouse sera (taken 3 months after immu-
nization) to ethanol-fixed normal human neutrophils. Goat anti-mouse conjugated to FITC was used as a
second antibody.
Histological studies
Histological examination of the lungs demonstrated several foci of perivascular mononuclear cell infiltrates consisting of monocytes and lymphocytes suggesting vasculitis (data not shown, see ref. 7); however, no giant cells or granulomas were found in the lungs. The lungs of the control mice did not show any pathological changes. There were no pathological findings in the kidneys of the mice immunized with human IgG-ANCA or in the controls.

Levels of cytokines in the mice immunized with hIgG-ANCA
Two weeks after boost immunization, cytokine concentrations were determined. Levels of IL-4, IL-6, and TNFα were significantly elevated in the mice immunized with hIgG-ANCA as compared with the controls (p = 0.04 for IL-4, p = 0.008 for IL-6, and p = 0.0003 for TNFα, Figs. 3A and 3B). In contrast, the levels of IL-2 and IFNγ were not different between the mice immunized with hIgG-ANCA and the controls (Fig. 3C). The levels of IL-1β were elevated in the mice immunized with hIgG-ANCA as compared with the controls, but this increase did not reach statistical significance (Fig. 3C).

Discussion
Lymphokines are regulatory peptides which are either produced by lymphocytes or which bind to lymphocytes to activate or suppress their function. Several lines of evidence support a pivotal role for cytokines in the initiation of autoimmunity. Studies have shown the unbalanced production of various cytokines in autoimmune diseases (9). Increased levels of interferons have been reported in rheumatoid arthritis (RA) (10), scleroderma (10), and myasthenia gravis (11). Additionally, increased TNF levels were reported in 25% of newly diagnosed IDDM (12), and in patients with active RA (13). In Graves’ disease significant increases in the mRNA levels of TNF, IL-1β, and IL-6 were reported in surgical specimens (14). Moreover, several recent studies have shown a beneficial effect for TNF blockade in RA (15), and Crohn’s disease (16). Similarly, in several animal models of auto-
In our experimental vasculitis model, which simulates human Wegener’s granulomatosis, the cytokine profile seemed to follow the Th2 pattern. This is especially interesting since recently high levels of IL-4, IL-6 and TNFα during the initial phase of the disease. This points to a pathogenic role for these cytokines in the development of the disease and suggests a preferential Th2 type response during the initiation of vasculitis in these mice. According to the Th1/Th2 paradigm, the cytokine profile of an immune response can characterize it as either Th1 or Th2 with regard to the T helper subset involved. This paradigm helps to explain the cellular basis for the diversity of T-cell responses seen in autoimmunity (18). Thus, Th1 cells, which promote inflammatory cellular immune responses, are biased towards the secretion of interferon-γ (IFN-γ), TNFα and IL-2, while Th2 cells, which induce humoral immunity, are biased towards the secretion of interleukins 4, 5, 6, 10 and 13 (19).

Autoimmune diseases with Th1 type cytokine profile are mainly organ-specific and include multiple sclerosis, Hashimoto’s thyroiditis, autoimmune (type 1) diabetes, primary biliary cirrhosis, and acute allograft rejection (20-23). In these diseases high levels of IL-2 and interferon-γ (IFN-γ) (Th1 pattern) were recorded, while IL-4, IL-6, and IL-10 (Th2 pattern) were low. Autoimmune diseases with the Th2 type cytokine pattern include graft versus host disease (GVHD), Ommen’s syndrome, and atopic disorders (22, 23). In these diseases high levels of IL-4, IL-6, and IL-10 (Th2 pattern) were recorded, while IL-2 and interferon-γ levels (Th1 pattern) were very low (22, 23). However, some diseases do not exactly fit these two cytokine profiles and may be regarded as heterogeneous cytokine type autoimmune diseases. These include systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), Sjögren’s syndrome, myasthenia gravis, and primary systemic vasculitis (24, 25). In these disorders high levels of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, TNFα) as well as other cytokines (interferon-γ, IL-2, IL-4, IL-10) were detected (26, 27).
Cytokines in experimental vasculitis / Y. Tomer et al.


