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Evidence in support of a self-perpetuating HLA-DR-dependent delayed-type cell reaction in rheumatoid arthritis

(HLA-DR antigens/synovial cells/collagen type II)

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ABSTRACT **Originating from observations on similarities between the rheumatoid synovial tissue and skin lesions in delayed-type hypersensitivity reactions—similarities as to massive infiltrates of "helper" T lymphocytes close to HLA-DR-expressing macrophage/dendritic cells—a notion is formed on the importance of local macrophage-dependent helper T-cell activation in the rheumatoid joint similar to that in a delayed-type skin reaction. *In vitro* studies on suspended synovial cells have been used to test and qualify these ideas. It is shown that (*i*) HLA-DR-expressing cells in normal synovial intima can, like epidermal Langerhans cells, mediate T-cell activation; (*ii*) the large numbers of rheumatoid synovial HLA-DR-expressing macrophage-like/dendritic cells are heterogeneous and mediate either efficient activation or suppression of T-lymphocyte proliferation, and (*iii*) specificity of rheumatoid T cells can be analyzed with the help of autologous synovial antigen-presenting cells; a specific anti-collagen type II response is reported in three patients.**

The immune reactions taking place in the synovial tissue in rheumatoid arthritis are poorly understood. Only for B lymphocytes and their secreted immunoglobulins have more extensive experiments on the cellular and molecular level been carried out (1).

However, new evidence now calls for detailed studies also on other immunocompetent cells, especially macrophage-like cells and T lymphocytes in the synovium; thus, T lymphocytes predominate among lymphocytes eluted from rheumatoid synovial tissue (2, 3), and macrophage-like and dendritic HLA-DR-expressing cells are abundant in rheumatoid synovial tissue as seen on both frozen sections and cell eluates (4). The close correlation between HLA-D/DR types and rheumatoid arthritis (5, 6)—coupled with the fact that phenotypes of the HLA-D-linked "immune response" gene appear to be expressed at the level where Ia (HLA-DR)-expressing macrophages present antigen to T lymphocytes (7)—emphasizes the need for further understanding of macrophage–T-cell interactions at the primary sites of this disease.

The above arguments have additional support from preliminary investigations, on both frozen sections and suspended cells from synovial tissue (8–10), that suggest a preference for macrophage–helper T-cell interactions within the rheumatoid synovial tissue. Thus, it appears that mechanisms similar to those operating in delayed-type cell reactions might be involved also in the pathogenesis of rheumatoid arthritis.

In this study we have subjected these ideas to experimental testing and qualification by (*i*) a comparative analysis between immunocompetent cells involved in a classical delayed-type hypersensitivity (DTH) skin reaction and cells present in the rheumatoid synovial tissue and (*ii*) a functional investigation of macrophage–T-cell interactions among synovial cells by using *in vitro* systems that permit analysis at the level of the antigen-presenting cells and that analyze the specificity of T-cell activation.

PATIENTS, MATERIALS, AND METHODS

Patients. The subjects comprised seven patients suffering from definite rheumatoid arthritis [American Rheumatism Association (ARA) criteria], who were subjected to therapeutic synovectomies; four patients with less severe rheumatoid arthritis from an outpatient clinic; five normal blood donors; and three healthy individuals vaccinated with bacillus Calmette–Guérin. Procedures for taking biopsies from skin and synovial tissues and for freeze-sectioning have been described (4, 11).

Antisera and Other Reagents. The rabbit antiserum against HLA-DR antigens and the IgG Fab fragments thereof have been described (12). Rabbit anti-alkaline phosphatase was provided by D. Y. Mason (13). Fluorescein isothiocyanate-conjugated and nonlabeled swine anti-rabbit IgG and fluorescein isothiocyanate-conjugated swine anti-mouse IgG were from Dakopatts (Copenhagen, Denmark). Biotin-treated horse anti-mouse IgG, avidin DH, and biotin-treated horseradish peroxidase ("ABC-kit") were from Vector Laboratories (Burlinggame, CA). The monoclonal antibodies denoted Leu 1, Leu 2a, Leu 3a were all provided by Becton–Dickinson (Sunnyvale, CA). Leu 1 defines all T cells (14), whereas Leu 2a defines a "suppressor/cytotoxic" T-cell subset and Leu 3a defines a "helper/inducer" T-cell subset (15).

Immunofluorescence and Immunocytochemical Staining Procedures. The different mouse or rabbit antibodies were used to stain suspensions of cells with the help of indirect immunofluorescence as described earlier (4). Frozen sections of tissue (4) were investigated by using a sensitive double immunoenzymatic technique that enables the simultaneous recognition of mouse and rabbit antibodies, which bind to the sections by means of peroxidase and alkaline-phosphatase-catalyzed reactions. The technique has been detailed (18) and is essentially a modification of a double immunoenzymatic technique originally described by Mason and Sammons (13), except that we have made use of the avidin–biotin–peroxidase complex (ABC) binding (16) instead of the unlabeled antibody peroxidase-antiperoxidase procedure.

Preparation of Cells from Peripheral Blood and from Normal and Rheumatoid Synovial Tissue. Peripheral blood lymphocytes were obtained by Ficoll flotation (17), and T cells were prepared from nonadherent cell fractions by rosetting techniques (18). Normal human synoviocytes were obtained by in-

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Abbreviations: PPD, purified protein derivative of tuberculin; DTH, delayed-type hypersensitivity.

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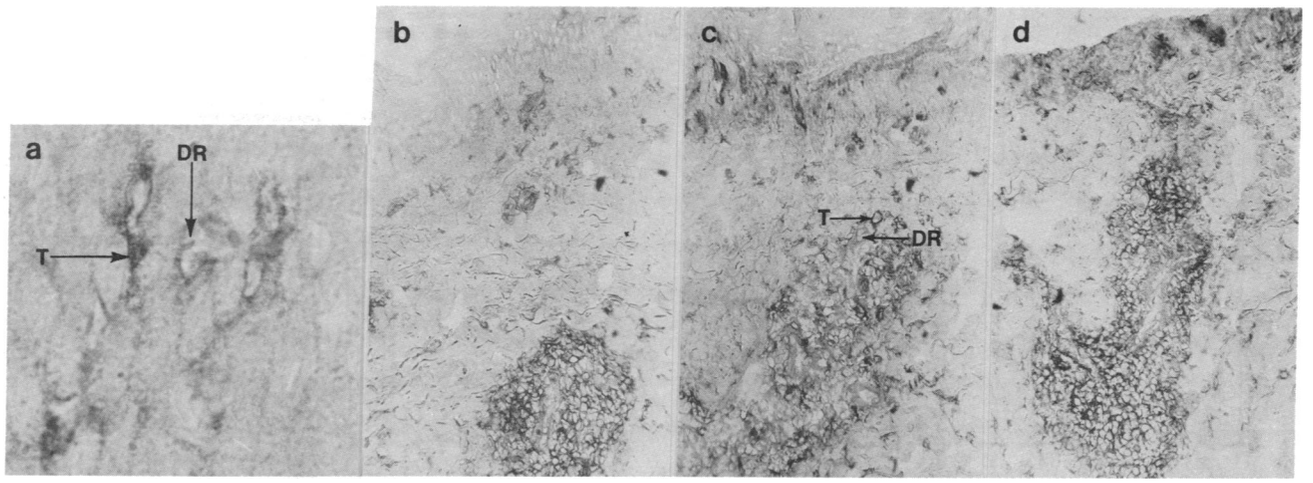

FIG. 1. Immunocytochemical double-staining technique applied on serial frozen sections from human skin DTH lesions 48 hr after intradermal injection of 0.1 ml (2 tuberculin units) of PPD. Combined staining was with rabbit anti-HLA-DR antibodies in all cases (blue, seen as light grey; see arrows in *a* and *c* designated DR). (*a* and *b*) Leu 1 (pan-T cells). (*c*) Leu 3a (helper). (*d*) Leu 2a (suppressor/cytotoxic) (brown, seen as dark grey; see arrows in *a* and *c* designated T). *b*, *c*, and *d* depict both epidermis and dermis, whereas *a* shows the epidermal portion at a higher magnification.

jection 3–6 hr postmortem of a trypsin-containing buffer into the knee joint for 15–20 min as described by Fraser and McCall (19). Preparation of cell suspensions from rheumatoid synovial tissues was by collagenase/DNase treatment as described (4, 20). Suspended cells were permitted to adhere to plastic dishes for 1–3 hr. The adherent cells were dissociated from the plastic by trypsin digestion and were used either directly or after fractionation on Percoll (Pharmacia, Uppsala, Sweden) density gradients (21). T cells were obtained by rosetting from the nonadherent fraction.

Mixed Leucocyte Culture Reaction and Antigen-Dependent T-Cell Stimulation. T-cell proliferation was determined by measuring [^{3}H]thymidine incorporation after stimulation with 2,500-rad irradiated autologous or allogeneic cells, with or without the presence of soluble antigens. The culture conditions and means of determining the responses were as described (4). For the antibody inhibition experiments, Fab fragments of normal rabbit serum and rabbit anti-HLA-DR antibodies were used in concentrations (0.05 mg/ml) that specifically inhibit allogeneic T-cell stimulation by peripheral blood lymphocytes (12).

Other Methods. Described methods for the demonstration of F_c-receptors (22) and staining for unspecific esterases (23, 24) were used. Collagen type II was prepared from bovine nose cartilage (25), and the purified protein derivative (PPD) of tuberculin was obtained from Statens Seruminstitut (Copenhagen, Denmark).

RESULTS

Similar Tissue Distribution of HLA-DR-Expressing Non-T- and T-Cell Subsets in Skin DTH Reaction and in Rheumatoid Synovial Tissue. In order to test the concept that presentation of antigens to helper T cells through HLA-DR-expressing synovial cells might be an important feature of rheumatoid arthritis, we chose to compare the situation in the rheumatoid joint with that of a well-characterized HLA-D/DR-dependent skin DTH reaction to PPD.

Thus, we first compared frozen sections of skin and synovial tissue for the tissue distribution of HLA-DR-expressing cells and subsets of T lymphocytes. This was accomplished by the use of a double immunoenzymatic technique permitting simultaneous visualization of bound rabbit anti-HLA-DR (alkaline phosphatase-catalyzed blue reaction) and mouse monoclonal antibodies against different T-cell subsets (peroxidase-catalyzed brown staining).

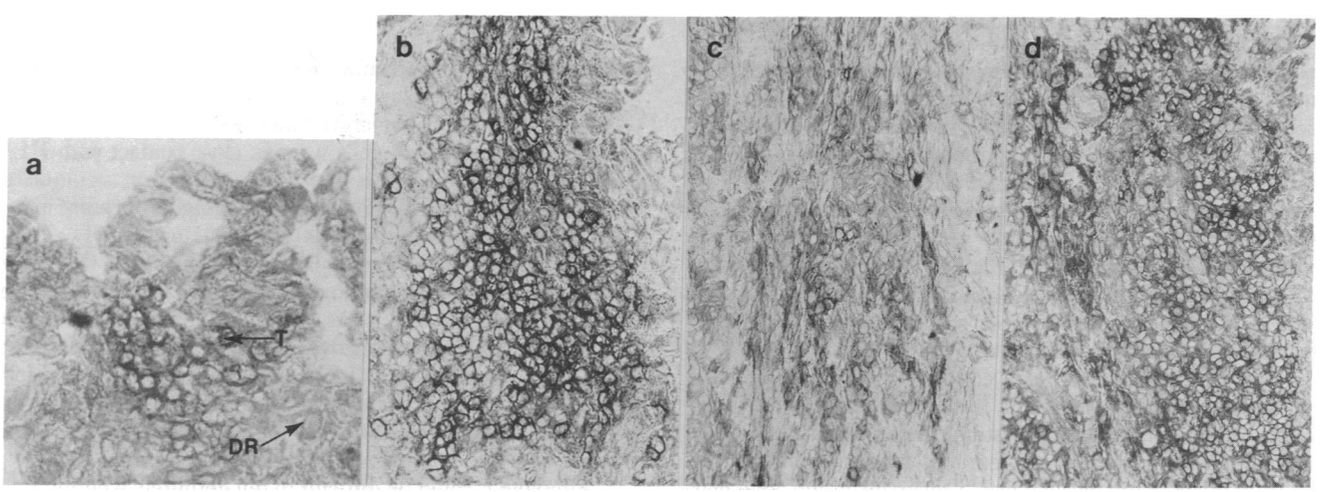

FIG. 2. Immunocytochemical double-staining technique applied on serial frozen sections from rheumatoid synovial tissue. Combined staining was with rabbit anti-HLA-DR antibodies in all cases (blue, seen as light grey; see arrow in *a* designated DR). (*a* and *b*) Leu 1 (pan-T cells). (*c*) Leu 3a (helper). (*d*) Leu 2a (suppressor/cytotoxic) (brown, seen as dark grey; see arrow in *a* marked T). *b*, *c*, and *d* depict a larger segment of the synovial tissue with synovial cavity upwards; *a* shows a section close to the synovial cavity at a higher magnification.

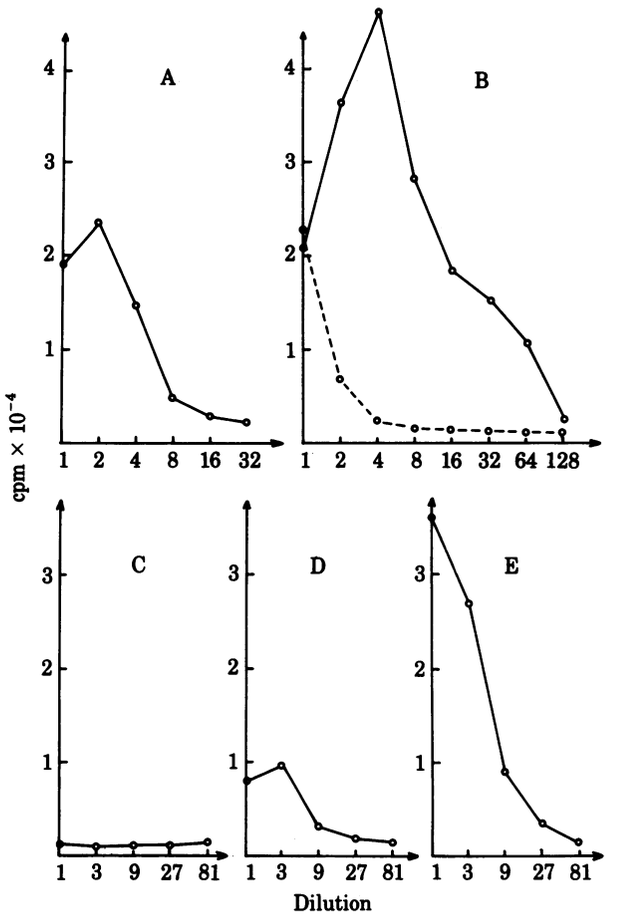

FIG. 3. Stimulatory capacity of graded numbers of 2,500-rad irradiated cells on the proliferative response of normal allogeneic T lymphocytes (10^6 cells per ml). (*A*) Normal human synoviocytes (1:1 dilution = 5×10^5 cells per ml). (*B*) Rheumatoid synovial adherent cells (1:1 dilution = 5×10^5 cells per ml) (○——○); non-T peripheral blood cells from the same rheumatic patient (1:1 dilution = 5×10^5 cells/ml) (○---○). (*C–E*) Fractions of the same population of rheumatoid adherent cells as in *B* after density gradient fractionation on discontinuous Percoll gradients (1:1 dilution = 2×10^5 cells/ml); the densities were: 1.0425/1.056 (interphase) (*C*), 1.056/1.0665 (interphase) (*D*), and 1.0665/1.0767 (interphase) (*E*). The data are means of triplicate determinations of the [^{3}H]thymidine incorporation, calculated as described (4) at 7 days of culture.

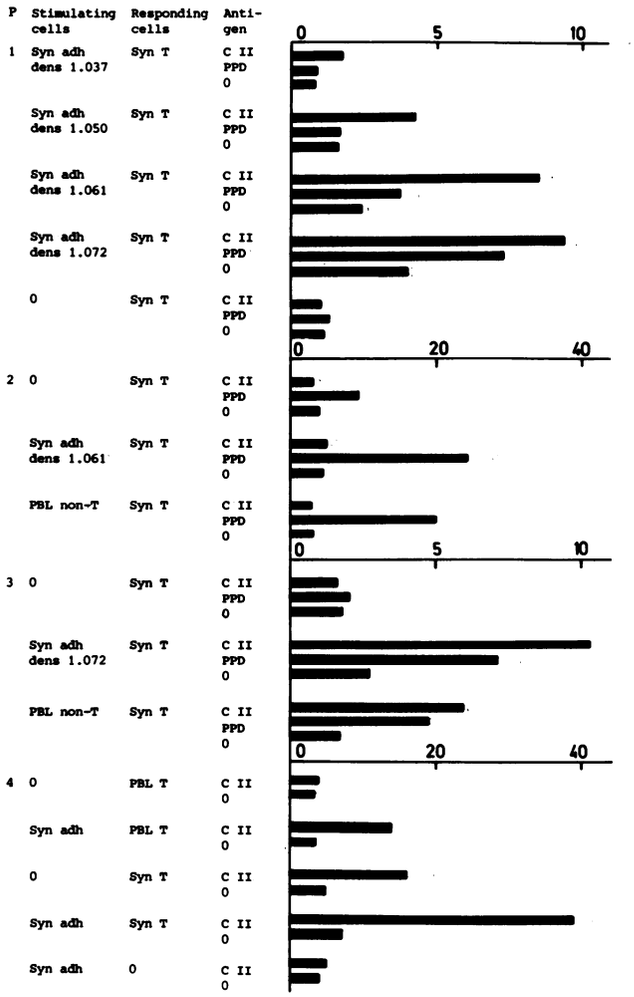

FIG. 4. Proliferation of 5×10^5 cells per ml of T lymphocytes from four rheumatoid patients (P) subjected to synovectomy, after coculture with different populations of 2,500-rad irradiated autologous cells and soluble antigens. PPD and collagen type II (C II) are used at a final concentration of 50 μg/ml. Different fractions of rheumatoid adherent synovial cells (Syn adh) were added at $2.5–10 \times 10^4$ cells per ml, and peripheral blood non-T cells (PBL non-T) were added at 2.5×10^5 cells per ml. The data are means of triplicate determinations of [^{3}H]thymidine incorporation calculated (4) at 5 days of culture and shown as cpm $\times 10^{-3}$.

Normal skin contains HLA-DR-expressing Langerhans cells in epidermis (11, 26) and HLA-DR-expressing macrophages and endothelial cells deeper in dermis; in an acute DTH reaction, such as that caused by PPD, a rapid invasion of T lymphocytes (Leu 1^+) is seen—most marked around small vessels (Fig. 1*b*) and in close contact with epidermal Langerhans cells (Fig. 1*a*). The vast majority of these T lymphocytes are of helper T-cell type (Leu $3a^+$) (Fig. 1*c*), and only very few are of suppressor/cytotoxic (Leu $2a^+$) phenotype (Fig. 1*d*). Thus, the distribution of T-cell subsets in this DTH reaction is different from that seen both in normal lymphoid tissues and in peripheral blood of healthy individuals (27, 28).

Normal synovial tissue also contains HLA-DR-expressing cells predominantly localized in the synovial intima (4). In addition, studies in the mouse system carried out with the help of bone-marrow chimeras suggest that such Ia-expressing synovial lining cells are derived from precursors in the bone marrow (29), which also has been shown for epidermal Langerhans cells (30).

Whereas almost no T lymphocytes were found in the normal human synovial tissue, all seven cases of rheumatoid synovial tissue that were investigated showed not only an abundance of non-T, macrophage-like, and dendritic cells (see ref. 4) but also a massive T-lymphocyte infiltration as shown by Leu 1 antibody binding. Most of these cells were in close contact with HLA-DR-expressing non-T cells; the T cells were often accumulated near the synovial cavity among cells that might represent in part proliferated synovial lining cells (Fig. 2*a*). However, T cells also were seen deeper in the membrane, and clusters of them were characteristically found around small vessels with HLA-DR-expressing endothelial cells. As was the case in the DTH skin reaction, helper (Leu 3a-reactive) T cells dominated widely over the suppressor/cytotoxic (Leu 2a-reactive) cells. Furthermore, the clusters of T cells in close apposition to HLA-DR-expressing non-T cells were mostly Leu $3a^+$, whereas the Leu 2a-reactive cells were often found more scattered in the tissue.

Although it could be difficult to tell definitely whether a single cell had stained simultaneously for peroxidase and alkaline phosphatase, it appeared that a large fraction of the T lymphocytes, both Leu $3a^+$ and Leu $2a^+$ cells, expressed HLA-DR antigens probably as a result of activation (30). Details on inves-

tigated rheumatoid patients and exact characteristics of immunohistological pictures will be reported elsewhere (8).

***In Vitro* Functional Studies on Interactions Between Synovial HLA-DR-Expressing Non-T Cells and T Lymphocytes.** In order to analyze the functional features of the cells of the synovias, suspended cells were obtained by enzyme-catalyzed dissociations of tissue—e.g., normal synovial intimal cells were obtained by postmortem trypsin injection into intact knee joints (19) and rheumatoid synovial cells were obtained by collagenase treatment of tissues from synovectomies (4). More than 90% of dissociated normal intimal cells are "macrophage-like," being positive for unspecific esterases, for adherence, and for possession of F_c receptors. On indirect immunofluorescence, these cells also express HLA-DR antigens, albeit with highly varying intensity. Likewise, most of rheumatoid synovial adherent cells express HLA-DR antigens but are Leu 1^- [fine details on their phenotypes are given elsewhere (4, 32)].

These synovial macrophage-like/dendritic HLA-DR-expressing cells were investigated for their T-cell activation properties as has been done for epidermal Langerhans cells and other types of macrophage-like cells (33–35). Normal synovial lining cells indeed can mediate an HLA-DR-dependent T-cell proliferation as measured in an allogeneic mixed leukocyte culture reaction (MLR) (Fig. 3*a*). However, their activating ability appeared to be lower than that of epidermal Langerhans cells (see also ref. 34).

In contrast, with rheumatoid synovial macrophage-like/dendritic cells, extremely few cells were needed for efficient allogeneic T-cell activation (see Fig. 3*b*). At the same time, this figure (representative for six investigated patients with rheumatoid arthritis) indicates heterogeneity among rheumatoid adherent cells as shown by the marked inhibition of T-lymphocyte proliferation with higher numbers of stimulating adherent cells. This heterogeneity can be demonstrated further by enriching adherent synovial cells in their enhancing or suppressive effects on T cells through density gradient separation (Fig. 3*c*). Extremely efficient T-cell-activating (mostly HLA-DR-expressing) cells were found in dense fractions, whereas large mostly HLA-DR^+ cells predominantly suppressing the T-cell response were present in the lighter fractions; when heavy and light fractions were mixed, the original biphasic pattern was reconstituted (not shown).

The T-cell enhancing "arm" of the adherent cells appears to work, like the normal synoviocytes, through T-cell recognition of HLA-DR antigens, as these reactions are specifically inhibitable by IgG Fab anti-HLA-DR antibodies (not shown). However, the working mechanism(s) of the suppressive "arm" is more unknown, although evidence from other systems suggest that soluble factors such as prostaglandins (36) might exert such effects.

Analysis of T-Cell Specificity. In systems such as epidermal Langerhans cells, the ability of HLA-DR-expressing cells to regulate allogeneic T-cell activation frequently parallels their capacity to "present" soluble or cell-bound antigens to helper T lymphocytes (34). This is also the case for rheumatoid synovial adherent cells.

Thus, rheumatoid T lymphocytes can respond specifically to both PPD and collagen type II antigens provided that appropriate synovial antigen-presenting cells are present (Fig. 4). Of the two tested antigens, PPD represents a well-characterized positive control antigen that is meant to define our experimental T-cell system (35). Cartilage-derived collagen type II, on the other hand, is currently studied with great interest as a possible autoimmunogen of importance in rheumatoid arthritis (37, 38). Three out of four synovectomized patients possessed collagen II-reactive T lymphocytes as measured by the proliferation assay. On the other hand, when unseparated peripheral blood lymphocytes from five normal blood donors and from four other rheumatics from an outpatient clinic were similarly tested in proliferation assays, good PPD responses but no collagen II responses were seen.

DISCUSSION

This study, aiming at an understanding of HLA-D/DR-dependent macrophage–T-cell interactions in rheumatoid disease, took as its starting point observations on such cells *in situ*, both in a classical skin DTH reaction and in rheumatoid synovial tissue.

In the skin, HLA-DR-expressing Langerhans cells and possibly other macrophage-like cells pick up antigen, in this case PPD, thereby enabling specific activation of T lymphocytes (33, 34). This is shown here to lead to a massive infiltration of helper T cells, whereas relatively few cytotoxic/suppressor T cells are seen.

The normal synovia contains, predominantly in the intima, HLA-DR-expressing macrophage-like cells that presumably are capable of picking up antigens for presentation to helper T cells. Therefore, the *in situ* picture of the rheumatoid synovia, demonstrating the close relationships between massive numbers of often activated helper T cells and HLA-DR-expressing macrophage-like cells, suggests that mechanisms similar to those operating in the classical DTH reaction are important also in inducing and maintaining rheumatoid arthritis. The distribution pattern of helper versus suppressor T cells in these two types of reactions is, thus, different from those observed in normal lymphoid tissues (27, 28). Unlike the acute DTH lesion, however, the chronically inflamed rheumatoid synovia contains an excessive number of HLA-DR-expressing non-T cells; in this respect the immunohistological picture of the synovia rather resembles that seen in chronic immune skin reactions, such as discoid lupus, lichen ruber (39), mycosis fungoides (40), or even graft versus host lesions (41, 42), in which a multitude of epidermal HLA-DR-expressing non-T cells are seen close to infiltrates of T lymphocytes. As to distribution of T-cell subsets in the rheumatoid tissue, the present data seem to agree with preliminary observations reported in a recent hypothesis article by Janossy *et al.* (9), albeit different techniques and different antibodies were used.

When functional studies on suspended synovial cells were initiated to study the immune mechanisms behind the lesions described by histology, it appeared that the basic prerequisites for a DTH reaction to occur in the synovia were fulfilled; thus, both normal intimal cells and adherent rheumatoid, macrophage-like cells could mediate HLA-DR-dependent T-cell activation. Whereas, the normal synoviocytes were moderately active as T-cell activators in their resting stage, the rheumatoid macrophage-like cell population contained extremely efficient T-cell activators and also cells that could suppress T-cell activity.

Thus, there appears to exist one enhancing and one suppressive arm among the adherent macrophage-like/dendritic cells. This finding might give clues as to the nature of possible suppressive phenomena taking place in the synovial tissue (43)—the level of T suppression is low (44)—but also should allow better analysis of autologous T-cell specificity in rheumatoid disease because the composition of synovial HLA-DR^+ antigen-presenting cells could be prepared to exclude suppressive effects and allow efficient antigen presentation (see Fig. 4).

It remains to be shown, though, which of these features are due to recruitment to the synovia by circulating cells and which are dependent on resident cells (e.g., intimal cells driven into proliferation and differentiation by factors released during the inflammatory process).

However, the main problems are (*i*) to elucidate the driving forces behind the recruitment of activated T lymphocytes and of the multitude of different macrophage-like/dendritic cells in the rheumatoid tissue and (*ii*) to understand how these cells relate to the destructive features of the disease. To address these questions, it should be necessary to postulate and test a possible series of events that lead gradually to the final lesion.

Thus, as a starting point, it seems obvious to postulate a local DTH reaction in the synovia in response to some antigen(s) (endogenous or exogenous). Given the presently defined experimental system, a number of antigens should be testable in this context; the anti-collagen type II responses found in the present preliminary study should be especially interesting for further studies because HLA-DRw4-related anti-collagen immune responses have recently been reported both in normal individuals and, to a higher extent, in rheumatoid subjects (38), and because anti-collagen type II immunity appears closely correlated to immune-response gene-linked collagen-induced arthritis in mice (45). Thus, we believe that the large numbers of helper T cells present in the rheumatoid synovial tissue might not represent necessarily a distorted balance between helper and suppressor T cells as compared to the situation in normal lymphoid tissue (9, 27, 28) but might merely represent a feature of a normal but perpetuated DTH reaction.

If this is so, it remains to postulate the mechanisms behind the emergence of large amounts of functionally heterogeneous HLA-DR-expressing macrophage-like/dendritic cells in the rheumatoid lesions. In the present system, little data are as yet available, but in other systems, activated T lymphocytes have been demonstrated to release factors that modulate functions and phenotypes of macrophage-like cells. Thus, resting HLA-DR$^-$ macrophages can be turned into HLA-DR-expressing cells (46) capable of either efficient enhancement or suppression of T-cell functions (36, 46), thereby apparently creating feedback circles involving macrophage-like cells.

As a tentative step in the pathogenesis lending some logic to the presence of large amounts of HLA-DR-expressing non-T cells in immune lesions, it is tempting to suggest that suppressive mechanisms within the macrophage system are triggered in response to a perpetuating T-cell activation. These activated macrophages might act by release of, for example, prostaglandins (36, 47) but simultaneously might release other factors such as collagenases (48) that possibly are involved both in perpetuating an immune reaction and in destroying the joints.

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