Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 9475–9479, December 1989
Immunology

Localization of a critical restriction site on the I-A_β chain that determines susceptibility to collagen-induced arthritis in mice

(major histocompatibility complex/class II genes/polymerase chain reaction/anticollagen autoantibodies/type II collagen-induced arthritis)

RIKARD HOLMDAHL*†, MIKAEL KARLSSON*, MIKAEL E. ANDERSSON*, LARS RASK‡, AND LEIF ANDERSSON§

*Department of Medical and Physiological Chemistry, Box 575, Uppsala University, Uppsala, Sweden; and Departments of ‡Cell Research and §Animal Breeding and Genetics, Swedish University of Agricultural Sciences, S-75123 Uppsala, Sweden

Communicated by Jan G. Waldenström, August 23, 1989

ABSTRACT **Type II collagen-induced arthritis (CIA) in mice is an autoimmune experimental model for rheumatoid arthritis. Susceptibility to CIA is associated with certain major histocompatibility complex class II haplotypes. The two very closely related haplotypes H-2^q and H-2^p differ in susceptibility to CIA. Only mice of H-2^q (DBA/1, B10G strains) but not mice of H-2^p-expressing strains (like strain B10P) develop CIA and an autoimmune response to type II collagen (CII) after immunization with CII. In contrast to H-2^p, the H-2^q haplotype does not express I-E molecules. The purpose of the present study was to identify, at the molecular level, the structures on major histocompatibility complex class II molecules determining susceptibility to CIA and CII responsiveness. We first excluded the possible suppressive involvement of E^p or A^p molecules by showing that F_1 hybrids between H-2^p and H-2^q haplotype strains, expressing E^p and A^p, are responders to CII and fully susceptible to CIA. Secondly, because A_α chains appear identical, we sequenced the A_β first-domain exons of p and q allotypes and found only four diverging amino acids in the predicted amino acid sequence. These variable residues were closely located at positions 85, 86, 88, and 89 at the end of the postulated α-helix, which is of importance for interactions with the antigenic peptide and the T-cell receptor. We suggest that this region is a critical major histocompatibility complex restriction site for CIA and CII responsiveness in H-2^q mice as compared with H-2^p mice. The CIA will now be an excellent autoimmune model for studies on interactions between autoantigenic peptide, autoreactive T cells, and a particular major histocompatibility complex molecule, as has been postulated to be the initial event also in rheumatoid arthritis.**

Immunization with type II collagen (CII) in mice, rats, and apes induces an autoimmune disease, with chronic and progressive development of arthritis, called the type II collagen-induced arthritis (CIA), which is widely used as a model for studies of rheumatoid arthritis (RA) (1–6). There are several similarities between RA and CIA. (*i*) A similar histopathology of the joints develops with occurrence of both marginal erosions and pannus formation as well as massive polymorphonuclear cell infiltration in acute lesions and edema formation in the synovium (3, 5, 7–9). (*ii*) Autoimmune reactions to both CII and IgG (rheumatoid factors) occur in both diseases (10–15). (*iii*) Susceptibility to both RA and CIA is sex-linked and is similarly influenced by female sex hormones and pregnancy (16–19). (*iv*) Both CIA and RA are influenced by genes within the major histocompatibility complex (MHC) (8, 20–23).

In the mouse, the susceptibility to arthritis after immunization with heterologous CII is restricted to the H-2^q and H-2^r haplotypes. With recombinant strains the susceptibility in the H-2^q haplotype has been mapped to the I-A locus in the MHC class II region (8). We have investigated the MHC restriction of the autoimmune response and development of arthritis after immunization not only with heterologous but also with autologous CII (20, 21). Immunization with autologous CII led to development of arthritis and/or anti-CII autoantibodies only in H-2^r, H-2^q, H-2^{w3}, and H-2^{w17} haplotype-carrying strains. The latter two haplotypes are derived from wild mice and are closely related to H-2^q (24–27). Immunization with various heterologous CII induced more severe arthritis in strains with H-2^q and H-2^r haplotypes and, in addition, induced an anti-CII autoantibody response but no arthritis in some strains with other haplotypes (20, 21). These findings suggest that the induction of arthritis was critically dependent on activation of autoreactive T cells, but that activation of T cells reactive with heterologous CII may enhance the severity and elevate the autoantibody response to CII. Furthermore, we found that B10P (H-2^p) mice responded to neither autologous nor heterologous CII in contrast to H-2 congenic B10G (H-2^q) mice and other mice of the H-2^q haplotype.

The difference in susceptibility to CIA between the H-2^q haplotype and the H-2^p haplotype is of particular interest because these haplotypes are closely related (24–27). The A^p and A^q molecules have been grouped into the same family by tryptic fingerprint analysis (24, 27). As for I-E molecules only E^p is expressed, whereas E^q is not expressed due to sequence abnormalities leading to defective RNA splicing of both E_α and E_β mRNA (28, 29). The purpose of the present study was to analyze the relative importance of A and E class II molecules and to determine the molecular basis for the observed association of CIA and CII autoimmunity with the MHC.

MATERIALS AND METHODS

Mice. DBA/1 and B10G mice, originally obtained from The Jackson Laboratory, and B10P mice, originally obtained from J. Klein (Tübingen, F.R.G.), were kept and bred in the animal unit at the Biomedical Center (Uppsala). Mice were used at an age of 8–10 weeks and age-matched in all experiments.

Collagens. Mouse CII from xiphoid cartilage and rat CII from a rat chondrosarcoma (30) were solubilized by pepsin digestion and subsequently purified as described by Miller (31). The collagens were stored lyophilized and dissolved in 0.1 M acetic acid before use.

Animal Experiments. Rat CII dissolved in 0.1 M acetic acid at a concentration of 1 mg/ml was emulsified in an equal volume of Freund's complete adjuvant (Difco) at 4°C, and 50

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: MHC, major histocompatibility complex; CIA, type II collagen-induced arthritis; CII, type II collagen; PCR, polymerase chain reaction; RA, rheumatoid arthritis.

†To whom reprint requests should be addressed.

μl of this emulsion was injected intradermally around the root of the tail. The clinical severity of arthritis was quantified according to a graded scale as described (3); 1 point = detectable swelling in one joint, 2 points = swelling in more than one but not all joints, 3 points = severe swelling of the entire paw and/or ankylosis. Each paw was graded, so each mouse could achieve a maximum score of 12 points. The mice were bled by retroorbital puncture, and the sera were stored at −70°C until used. The quantification of anti-CII reactive autoantibodies in sera was performed with an enzyme-linked immunosorbent assay (ELISA) using mouse CII on the solid phase as described in detail (20).

Polymerase Chain Reaction (PCR) Analysis and DNA Sequencing. Genomic DNA samples from B10G and B10P strains were prepared from liver tissue according to the following procedure: Liver tissue was ground to a fine powder in liquid nitrogen. The cell powder was then mixed with 40 ml of lysis buffer (1% Triton X-100 in 0.32 M sucrose/1 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$). The mixture was centrifuged at 1500 × *g* for 10 min at 4°C. The pellet was resuspended in 10 mM Tris·HCl, pH 8.0/400 mM NaCl/2 mM EDTA/0.5% SDS/proteinase K at 0.2 mg/ml and incubated at 37°C overnight. Protein contaminants were removed by precipitation with a saturated NaCl solution (32). DNA was then recovered by ethanol precipitation.

Four primers for PCR analysis were synthesized on an Applied Biosystems 380A oligonucleotide synthesizer; LA1, 5′-ATAGGATCCTGACCGCGTCCGTCCGCAG-3′; LA2, 5′-GAGAATTCACCAAGCCGCCGCAGGGA-3′; LA3, 5′-GCGGATCCACCAACGGGACGCA-3′; LA4, 5′-ACGAATTCCTCCCGGTTGTAGATGT-3′. The PCR was carried out with 2.5 units of thermostable *Thermus aquaticus* (*Taq*) polymerase (Perkin–Elmer/Cetus) according to the manufacturer's recommendation in a final volume of 50 μl. Analysis was done with 1 μg of genomic DNA and oligonucleotide primers at a concentration of 1 μM. Denaturation occurred at 93°C for 30 sec, hybridizing at 55°C for 1 min, and extension at 72°C for 2 min. Single-stranded DNA for direct sequencing was generated by asymmetric PCR by using one of the primers at a concentration of 1 μM and the other at 0.02 μM (33). DNA sequencing was then performed with the latter primer by using the dideoxy chain-termination method with [α-^{35}S]dATP and the modified phage T7 DNA polymerase (United States Biochemical) (34).

RESULTS

Dominant Influence by A_β Chain on the Susceptibility to CIA. The different susceptibility to CIA and the different ability to produce anti-CII antibodies exhibited by mice of H-2^q and H-2^p haplotypes might either be due to a dominant positive influence of A^q or a negative influence of E^p molecules. The latter mechanism has been described in other systems in which expression of certain I-E alleles mediated a dominant suppression in F_1 hybrid mice (35, 36). To resolve this question F_1 hybrid mice of H-2^p and H-2^q haplotypes were analyzed (Table 1). In accordance with earlier observations (17, 20, 21), DBA/1 (H-2^q) mice developed CIA with higher incidence and severity compared with B10G (H-2^q), suggesting an influence of non-MHC B10 background genes. For these reasons we investigated F_1 hybrid mice by using both B10G and DBA/1 as parental strains combined with B10P (H-2^p). The result clearly shows that expression of E^p or A^p molecules does not have a suppressive influence on the development of CIA or on the anti-CII autoantibody response.

Thus, only a dominant influence of A^q can explain the MHC class II association of CIA development and CII responsiveness. Furthermore, the I-A restriction is most likely dependent on the A_β molecule because the extracellular domains of A^p_α and A^q_α displayed identical patterns when compared by tryptic fingerprint pattern, whereas the extracellular portions of A^p_β and A^q_β chains differed in 5 of 28 peptides (24). Because polymorphic sites of functional importance have been found only in the first domain of class II molecules (37, 38), we decided to determine the structure of this region of the A^q_β and A^p_β molecules.

Identification of a Critical Restriction Site for the Susceptibility to CIA on the A_β Molecule. To determine the amino acid sequence of the A_β first domain of H-2^q and H-2^p the corresponding exon (exon 2) was amplified by PCR and sequenced. An attempt to amplify the complete first-domain exon by using the primers LA1 and LA2 was not successful for unknown reasons. Two internal primers (LA3, LA4) were then synthesized, and the exon was successfully amplified in two parts with the two primer pairs LA1/LA4 and LA2/LA3. One of the external primers (LA2) was placed partly in the exon (Fig. 1). However, substitutions in the sequence covered by this primer are unlikely, especially when taken into account the lack of polymorphism in the particular region. The A^p_β (B10P) and A^q_β (B10G) nucleotide sequences of exon 2 were determined by direct sequencing of PCR-amplified DNA (Fig. 1). A comparison of the p and q sequences reveals differences only at the codons for amino acid positions 85, 86, 88, and 89. The deduced amino acid differences (Fig. 2) show that the A^q_β chain has a more positive charge in this region as compared with the A^p_β chain; glycine instead of glutamic acid at codon 85 and histidine instead of proline at codon 89.

DISCUSSION

We demonstrated that the presence of only four amino acid substitutions at positions 85, 86, 87, and 89 in the A_β molecule can explain the different CIA susceptibility and CII immune responsiveness of H-2^q and H-2^p haplotype mouse strains. This very limited difference between the I-A molecules of q and p origin may explain the earlier difficulties to isolate monoclonal antibodies and alloreactive T-cell clones that distinguish I-A^p and I-A^q (24–27). However, an alloreactive response has been recorded with secondary T-cell cultures

Table 1. Arthritis development and autoantibody response in male mice 12 weeks after rat CII immunization

Strain*	H-2		*n*	Incidence of arthritis, %	Mean severity of arthritis	Serum anti-CII autoantibodies, mean μg/ml ± SD†
	I-A	I-E				
B10P	p	p	10	0	0	1
B10G	q	—	10	30	3.7	35
DBA/1	q	—	10	100	7.1	88
(DBA/1 × B10P)F_1	q/p	p	11	73	7.4	200 ± 97
(DBA/1 × B10G)F_1	q	—	10	90	9.9	224 ± 59
(B10G × B10P)F_1	q/p	p	9	33	3.6	172 ± 59

*Data from the parental strains are from an earlier described experiment (20).
†Autoantibody levels in serum from parental strains were determined from pooled serum samples collected 15 weeks after immunization. Therefore, the SD of the biological variation cannot be given for these strains.

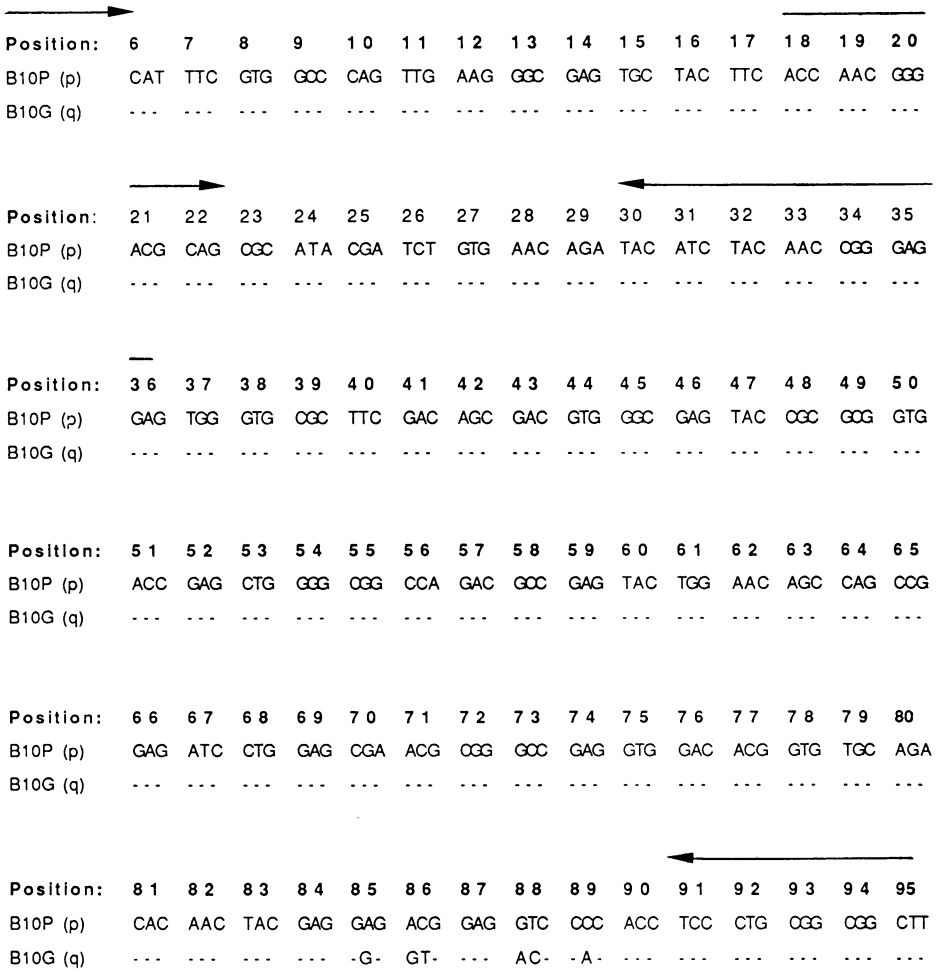

FIG. 1. Nucleotide sequence of the A_β first domain exon from B10P (H-2^p) and B10G (H-2^q). The positions for the PCR primers used are indicated by arrows.

derived from I-A^p xI-A^k mice [(C3H.NB × B10.AQR)F_1] and stimulated with spleen cells expressing A^q [from B10T(6R)] (24). This response can now be explained by recognition of structures involving the 85–89 region of the A_β molecules.

Comparisons of large number of A_β sequences from different haplotypes show that the 85–89 region contains a relatively high degree of variability (Fig. 2). In a three-dimensional model of the A_β molecule, this region is suggested to be located at the end of an α-helix of importance for the specific interactions between the T-cell receptor, the MHC molecule, and the antigenic peptide (38). Although the 85–89 region is the critical restriction site for the CIA susceptibility and CII immune responsiveness of the H-2^q haplotype mice compared with H-2^p haplotype mice we do not know whether the q-specific residues are of importance for direct binding of the antigenic peptide, for orientation of the peptide allowing interaction with the T-cell receptor and the bound peptide and/or for direct binding by the T-cell receptor. These interactions may now be elucidated using T-cell clones from H-2^q haplotype mice, peptides from the CII molecule, and antigen-presenting cells from H-2^q and H-2^p haplotype mice. Furthermore, the structures presented in the 85–89 region of the A^q_β molecule may have important influence on the selection of the T-cell repertoire in the thymus, allowing potentially arthritogenic T cells to be produced in H-2^q strains but not in H-2^p strains. These questions can now be addressed by analysis of mice made transgenic with site-directed mutants of the A^q_β molecules.

However, the I-A^q specific region in positions 85–89 is by no means sufficient for the specific interactions between the putative CII peptide, the T-cell receptor, and the class II molecule. Many residues in both A_α and A_β molecules participate in these interactions, and we have summarized (Fig. 2) known amino acid sequences translated from the present, as well as previously established nucleotide sequences of A_β first-domain exons of different haplotypes. This summary shows that only the A^s_β haplotype has an identical sequence with A^q_β in the 85–89 region. However, the A^s_β sequence has many differences as compared with A^q_β sequences at other positions, and there may be differences also in the A_α chain (42, 43), which could explain why H-2^s haplotype strains are not susceptible to CIA or develop a CII autoimmune response (8, 20, 21). Nevertheless, it is of interest to note that in some other cases immune responses are restricted to both H-2^s and H-2^q. One example is the encephalitogenic response of peptide 88–100 of the myelin basic protein that is inducible in SJL (H-2^s) and SWR (H-2^q) mice (44). Another example is the specific T-cell proliferative response to a GT ($Glu_{50}Tyr_{50}$) polymer (45). In this case I-A^q and I-A^s determine high responsiveness and I-A^p low responsiveness, and a possible suppressive influence of I-E^p could be excluded. Thus, also in the immune response to the GT polypeptide and possibly the induction of chronic experimental allergic encephalomyelitis in SJL and SWR mice a critical MHC restriction site may be located in the 85–89 region of A^q_β and A^s_β.

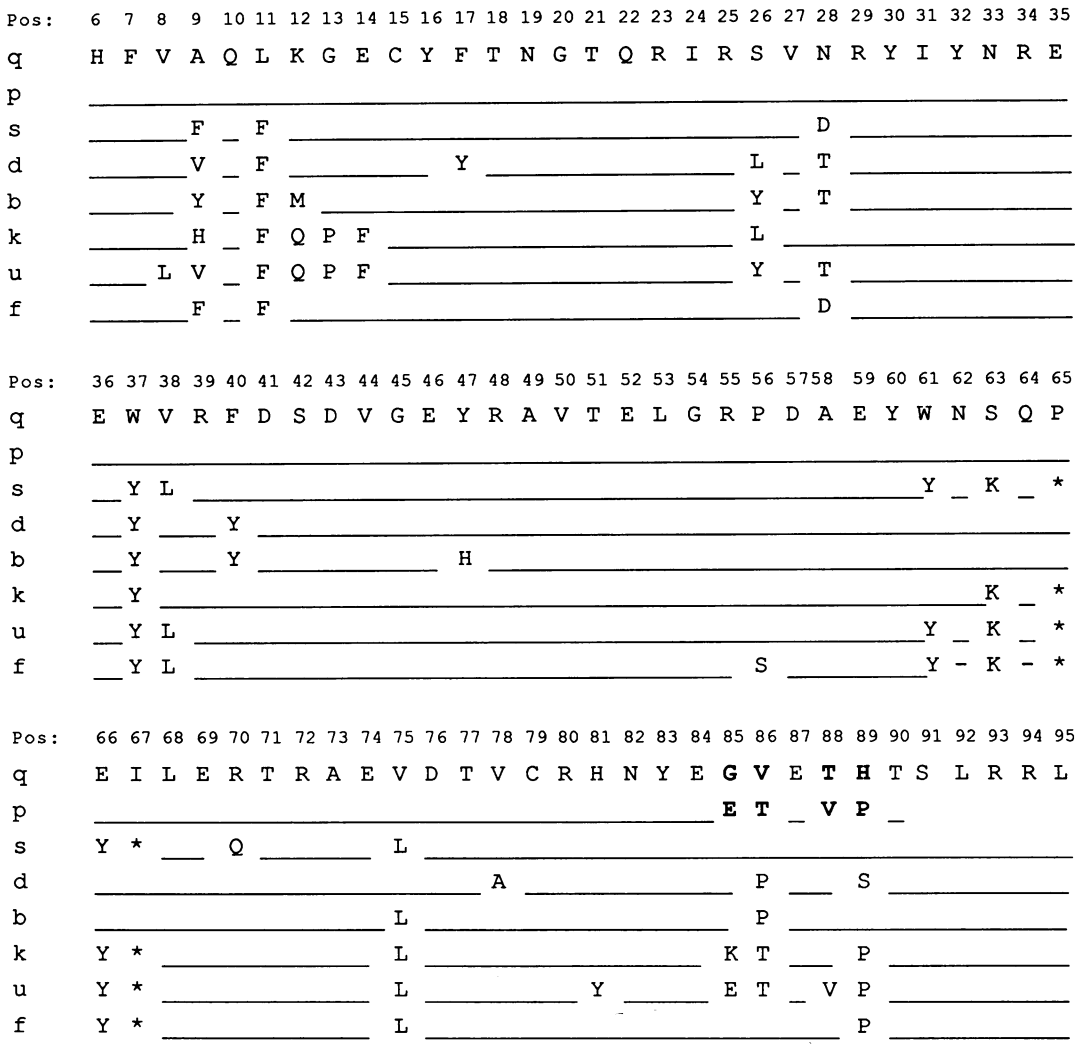

FIG. 2. Translated amino acid sequence (in one-letter code) corresponding to the A_β first-domain exon of various haplotypes. The s, k, u, f (39), the b (40), and the d (41) sequences have been published earlier. The p and q sequences are from the present investigation; diverging amino acids are in boldface. The q sequence from the present investigation was determined from B10G mice and an earlier published q (39) determined from DBA/1; these were found to be identical except for a silent mutation at codon 70. *, Deletion of the residue at the indicated position (Pos).

RA is associated with HLA-DR4 region in linkage disequilibrium with HLA-DQw3 (23). The strongest association is with the Dw4, Dw13, Dw14, and Dw15 subtypes of DR4, which have sequence similarities at positions 70 and 71 of the DRB chain (46). From these findings it has been suggested that most patients with RA suffer from an immunospecific disease dependent on recognition of one particular self peptide bound to a particular DR restriction site and recognized by a particular autoreactive T cell (23). Thus, RA and CIA may share this kind of etiological process, although this does not necessarily mean that an identical autopeptide is bound to similar structures on class II molecules and presented to a T cell with an identical T-cell receptor in the two diseases. Rather, the CIA model will be valuable to elucidate the pathogenetic mechanisms leading to autoimmune arthritis, which may be shared with RA, and to develop new therapeutic strategies using specific peptides of importance for the initial recognition between autoreactive T cells and autoantigens.

This work was supported by the Swedish Medical Research Council, Swedish Research Council for Forestry and Agriculture, Swedish Cancer Society, Craaford Foundation, Nanna Swartz Foundation, King Gustaf V:s 80-years Foundation and Riksförbundet mot Reumatism.

1. Courtenay, J. S., Dallman, M. J., Dayan, A. D., Martin, A. & Mosedal, B. (1980) *Nature (London)* **283,** 666–667.
2. Holmdahl, R., Jansson, L., Gullberg, D., Rubin, K., Forsberg, P. O. & Klareskog, L. (1985) *Clin. Exp. Immunol.* **62,** 639–646.
3. Holmdahl, R., Jansson, L., Larsson, E., Rubin, K. & Klareskog, L. (1986) *Arthritis Rheum.* **29,** 106–113.
4. Trentham, D. E., Townes, A. S. & Kang, A. H. (1977) *J. Exp. Med.* **146,** 857–868.
5. Larsson, P., Kleinau, S., Holmdahl, R. & Klareskog, L. (1989) *Arthritis Rheum.*, in press.
6. Yoo, T. J., Kim, S. Y., Stuart, J. M., Floyd, R. A., Olson, G. A., Cremer, M. A. & Kang, A. H. (1988) *J. Exp. Med.* **168,** 777–782.
7. Zvaifler, N. J. (1973) *Adv. Immunol.* **16,** 265–336.
8. Wooley, P. H., Luthra, H. S., Stuart, J. M. & David, C. S. (1981) *J. Exp. Med.* **154,** 688–700.
9. Holmdahl, R., Jonsson, R., Larsson, P. & Klareskog, L. (1988) *Lab. Invest.* **58,** 53–60.
10. Jasin, H. E. (1985) *Arthritis Rheum.* **28,** 241–248.
11. Posner, M. R., Barrach, H. J., Elboim, H. S., Nivens, K., Santos, D. J., Chichester, C. O. & Lally, E. V. (1989) *Hybridoma* **8,** 187–197.
12. Londei, M., Savill, C. M., Verhoef, A., Brennan, F., Leech, Z. A., Duance, V., Maini, R. N. & Feldmann, M. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 636–640.
13. Holmdahl, R., Nordling, C., Rubin, K., Tarkowski, A. & Klareskog, L. (1986) *Scand. J. Immunol.* **24,** 197–203.
14. Holmdahl, R., Tarkowski, A., Nordling, C., Rubin, K. &

Klareskog, L. (1987) *Monogr. Allergy* **22,** 71–80.
15. Punjabi, C. J., Wood, D. D. & Wooley, P. H. (1988) *J. Immunol.* **141,** 3819–3822.
16. Vandenbroucke, J. P., Valkenburg, H. A., Boersma, J. W., Cats, A., Festen, J. J. M., Huber-Bruning, O. & Rasker, J. J. (1982) *Lancet* **ii,** 839–842.
17. Holmdahl, R., Jansson, L. & Andersson, M. (1986) *Arthritis Rheum.* **29,** 1501–1509.
18. Hench, P. S. (1938) *Mayo Clin. Proc.* **13,** 161–167.
19. Waites, G. T. & Whyte, A. (1987) *Clin. Exp. Immunol.* **67,** 467–476.
20. Holmdahl, R., Klareskog, L., Andersson, M. & Hansen, C. (1986) *Immunogenetics* **24,** 84–89.
21. Holmdahl, R., Jansson, L., Andersson, M. & Larsson, E. (1988) *Immunology* **65,** 305–310.
22. Stastny, P. (1978) *N. Engl. J. Med.* **298,** 869–871.
23. Gregersen, P. K., Silver, J. & Winchester, R. J. (1987) *Arthritis Rheum.* **30,** 1205–1213.
24. Peck, A. B., Darby, B. & Wakeland, E. K. (1983) *J. Immunol.* **131,** 2432–2439.
25. Peck, A. B., Smith, R. T. & Jadus, M. R. (1983) *J. Immunol.* **130,** 2067–2070.
26. Shaut, D. M., Wakeland, E. K., Maurer, P. H. & Peck, A. B. (1984) *J. Immunol.* **133,** 1410–1416.
27. Wakeland, E. K. & Klein, J. (1983) *J. Immunol.* **130,** 1280–1286.
28. Mathis, D. J., Benoist, C., Williams, V. E., II, Kanter, M. & McDevitt, H. O. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 273–277.
29. Tacchini-Cottier, F. M. & Jones, P. P. (1988) *J. Immunol.* **141,** 3647–3653.
30. Smith, B. D., Martin, G. R., Dorfman, A. & Swarm, R. (1975) *Arch. Biochem. Biophys.* **166,** 181–186.
31. Miller, E. S. (1972) *Biochemistry* **11,** 4901–4909.
32. Miller, S. A., Dykes, D. D. & Polesky, H. F. (1988) *Nucleic Acids Res.* **16,** 1215–1215.
33. Gyllensten, U. B. & Erlich, H. A. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 7652–7656.
34. Tabor, S. & Richardson, C. C. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 4767–4771.
35. Nagy, Z. A., Baxevanis, C. N. & Klein, J. (1982) *J. Immunol.* **129,** 2608–2611.
36. Wassom, D. L., Krco, C. J. & David, C. S. (1987) *Immunol. Today* **8,** 39–43.
37. Germain, R. N., Ashwell, J. D., Lechler, R. I., Margulies, D. H., Nickerson, K. M., Suzuki, G. & Tou, J. Y. L. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 2940–2944.
38. Brown, J. H., Jardetzky, T., Saper, M. A., Samraoui, B., Bjorkman, P. J. & Wiley, D. C. (1988) *Nature (London)* **332,** 845–850.
39. Estess, P., Begovich, A. B., Koo, M., Jones, P. P. & McDevitt, H. O. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 3594–3598.
40. Larhammar, D., Hammerling, U., Denaro, M., Lund, T., Flavell, R. A., Rask, L. & Peterson, P. A. (1983) *Cell* **34,** 179–188.
41. Malissen, M., Hunkapiller, T. & Hood, L. (1983) *Science* **221,** 750–753.
42. Benoist, C. O., Mathis, D. J., Kanter, M. R., Williams, V. E., II, & McDevitt, H. O. (1983) *Cell* **34,** 169–177.
43. Landais, D., Matthes, H., Benoist, C. & Mathis, D. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 2930–2934.
44. Fritz, R. B., Skeen, M. J., Chou, C.-H. J., Garcia, M. & Egorov, I. K. (1985) *J. Immunol.* **134,** 2328–2332.
45. Vidovic, D., Klein, J. & Nagy, Z. A. (1985) *J. Immunol.* **134,** 3563–3568.
46. Gregersen, P. K., Shen, M., Song, Q., Merryman, P., Dagar, S., Seki, T., Maccari, J., Goldberg, D., Murphy, H., Schwenzer, J., Wang, C. Y., Winchester, R. J., Nepom, G. T. & Silver, J. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 2642–2646.