Cellular hyperimmunoreactivity to rubella virus synthetic peptides in chronic rubella associated arthritis

L A Mitchell, D Décarie, R Shukin, A J Tingle, D K Ford, M Lacroix, M Zrein

Abstract

**Objectives**—Immune recognition of the major structural proteins of rubella virus by peripheral blood mononuclear cells and synovial inflammatory infiltrates of a patient with documented chronic rubella associated arthritis was compared with responses of normal healthy rubella virus immunoreactive subjects to establish if there were unusual response patterns associated with rubella associated arthritis in this subject.

**Methods**—Synthetic peptides (16–33 amino acids in length) representing selected amino acid sequences of the rubella virus envelope (E1 and E2) and capsid (C) proteins were used in lymphocyte stimulation assays with peripheral blood mononuclear cells or synovial inflammatory infiltrates to determine T lymphocyte recognition of antigenic sites within the synthetic peptides. A rubella virus specific polymerase chain reaction was used to determine the persistence of rubella virus in the patient’s cells.

**Results**—The patient’s peripheral blood mononuclear cells showed abnormally increased lymphoproliferative responses to three E1 synthetic peptides encompassing residues 219–234, 389–411, and 462–481, and one E2 synthetic peptide containing the sequence 50–72, of which the last three were predicted to contain T cell antigenic sites. Although the patient’s peripheral blood mononuclear cells showed positive proliferative responses to C synthetic peptides, these were not unusual. The number of synthetic peptides within the E1, E2, and C panels recognised by the patient’s peripheral blood mononuclear cells was greater than was previously observed in normal healthy subjects. The recognition of synthetic peptides by synovial inflammatory infiltrates was similar to peripheral blood mononuclear cells but the responses measured were lower. The polymerase chain reaction was negative for rubella virus detection in peripheral blood mononuclear cells and synovial inflammatory infiltrates.

**Conclusions**—Abnormally increased T cell recognition of antigenic sites within rubella virus E1 and E2 proteins observed in this patient with rubella associated arthritis suggests chronic antinuclearaemia due to persistent rubella virus in tissue sites other than peripheral blood mononuclear cells or synovial inflammatory infiltrates.

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The association of rubella virus with the development of joint manifestations (arthritis or arthralgia, or both) after natural infection or vaccination, particularly in adult women, has long been recognised. Rubella associated arthropathy is thought to be due to an underlying defect in the protective immune response leading to the persistence of the rubella virus in tissue sites such as the synovium with either immunologically or virally mediated tissue damage. The isolation of rubella virus from peripheral blood mononuclear cells or synovial inflammatory cells from some adult patients with rubella associated arthritis supports this hypothesis. It remains unclear, however, if there are specific immunological defects associated with rubella associated arthritis.

Reports of low levels of antibody reactive with a synthetic peptide representing a newly defined neutralisation domain of the rubella virus E1 envelope protein suggest that patients with congenital rubella syndrome, those in whom rubella virus immunisation failed, and certain patients with rubella associated arthritis may have specific defects in immune recognition. It is still unclear whether the antibody is the sole protective mechanism in rubella and T lymphocyte responses have been measured in human cells stimulated with whole rubella virus or synthetic peptides representing defined amino acid sequences of the rubella virus envelope (E1, E2) and capsid (C) proteins. This paper reports the case of an adult woman with chronic rubella associated arthritis who has been the subject of two previous papers. We report here the results of a new study in which peripheral blood mononuclear cells and synovial inflammatory infiltrates of this patient were tested in lymphocyte stimulation assays with synthetic peptides representing selected sequences of rubella virus E1, E2, and C proteins, to determine if there were unique immunoreactivities associated with her disorder.
Methods

CASE OVERVIEW

At the time of study the patient was a 73 year old woman with a 19 year history of persistent inflammatory polyarthritis, which began about 12 years after she contracted rubella from a family member.2 Sequential laboratory studies of rubella virus specific cellular immunity in peripheral blood mononuclear cells and synovial inflammatory infiltrates from this patient showed abnormally increased lymphoproliferative reactivity to rubella virus antigens compared with 21 other microbial antigens,7 8 including yersinia and parvovirus, which have also been shown to play a part in infectious arthritides. Sequential studies of serum and synovial fluid showed that, though the patient had substantial levels of rubella virus specific antibody detectable by whole virus enzyme linked immunosorbent assays (ELISAs) and immunoblot assays, she did not have the antibody reactive with the E1 neutralisation domain synthetic peptide.3 8

DETECTION OF RUBELLA VIRUS GENOMIC SEQUENCES

Rubella virus specific polymerase chain reaction amplification was performed as previously described (Mitchell L A et al, unpublished results) after acid guanidine extraction of total cellular RNA from either peripheral blood mononuclear cells or synovial inflammatory infiltrates, and random hexanucleotide priming of complementary DNA (cDNA) using MMTV reverse transcriptase. Specific polymerase chain reaction primers were constructed from the codified nucleotide sequence of the rubella virus E1 protein.9 After 35-40 cycles of amplification the polymerase chain reaction products were subjected to ethidium bromide-agarose gel electrophoresis to identify specific rubella virus product bands. The sensitivity of polymerase chain reaction detection of rubella virus by these methods is estimated to be less than 100 gene copies per 5 μg cDNA.

RUBELLA VIRUS SYNTHETIC PEPTIDES

Synthetic peptides (16-33 amino acids in length) were prepared by automated solid phase synthesis10 from predicted amino acid sequences of the E1, E2, and C proteins of Therien,8 11 wild type M33,12 and RA27/3 vaccine13 rubella virus strains by BioChem Immunosystems, Laval, Quebec, Canada. After purifying the synthetic peptides by reversed phase high performance liquid chromatography and verifying their composition by amino acid analysis, the synthetic peptides were tested in lymphoproliferation assays as described in the following section. The synthetic peptides that were selected for study and their relative sequence positions in the three rubella virus structural proteins are listed in the table.

LYMPHOCYTE STIMULATION ASSAYS

During a follow up examination 60 ml heparinised blood was collected by venepuncture. At that time approximately the same volume of synovial fluid was aspirated. Peripheral blood mononuclear cells or synovial inflammatory infiltrates were isolated from synovial fluid by density gradient centrifugation and used at cell densities of 100 000 cells/well (0-3 ml) in lymphocyte stimulation assays using rubella virus E1, E2, and C synthetic peptides at a final concentration of 15 μg/ml as previously described.6 7 Heat inactivated rubella virus (106 pfu) or phytohaemagglutinin (15 μg/ml) were added to a separate series of wells as stimulation (positive) controls, whereas negative (background) control wells received only medium supplemented with autologous plasma. All analyses were performed in triplicate. Results were expressed as the stimulation index, which was determined by dividing the mean 3H counts incorporated in the presence of stimulating peptide, rubella virus, or phytohaemagglutinin by the mean of the background counts in negative wells. Stimulation indices greater than or equal to 2 were considered positive and were verified for all positive results (p<0.05) by performing two tailed t tests of the mean gross counts per minute obtained with synthetic peptide or rubella virus stimulated and unstimulated wells.
Results
POLYMERASE CHAIN REACTION DETECTION OF RUBELLA VIRUS GENOMIC SEQUENCES
Although positive rubella virus controls contained specific bands at the appropriate position after ethidium bromide-agarose gel electrophoresis, repeated polymerase chain reaction using cDNA prepared from RNA extracted from either the patient’s peripheral blood mononuclear cells or synovial inflammatory infiltrates cells did not identify rubella virus in the samples collected at this time.

LYMPHOCYTE STIMULATION ASSAYS
Stimulation of peripheral blood mononuclear cells with E1 synthetic peptides (table and fig 1, E1) showed positive responses with 10/16 peptides tested. Particularly strong proliferative responses were observed with synthetic peptides E1-7 (219–239), E1-15(389–411A), and E1-17(462–481), which resulted in stimulation indices that were three to seven fold higher than those observed in positive lymphoproliferative responses to these synthetic peptides observed with peripheral blood mononuclear cells from normal subjects. A high stimulation index was also observed with the synthetic peptide E1-12(234–252); however, this value was within the range of standard indices reported in normal healthy subjects. Stimulation indices observed when synovial inflammatory infiltrates were tested with E1 synthetic peptides were lower than stimulation indices measured with peripheral blood mononuclear cells (fig 2, E1) and not all synthetic peptides were tested owing to insufficient numbers of cells. In general, however, positive responses of synovial inflammatory infiltrates were observed with the same synthetic peptides as with peripheral blood mononuclear cells, with the exception of peptides E1-12(234–252) and E1-16(389–412W).

Figure 1 Comparative lymphoproliferative responses of patient and normal control subject peripheral blood mononuclear cells to rubella virus synthetic peptides. Freshly isolated patient peripheral blood mononuclear cells (hatched bars) and peripheral blood mononuclear cells from 48 healthy adult donors (solid bars) were incubated with heat inactivated whole rubella virus (M33 strain) or synthetic peptides representing selected amino acid sequences of rubella virus E1, E2, and C proteins as shown in the table. Histogram bars represent the arithmetic mean of stimulation indices measured in the normal subject group compared with the response measured for patient peripheral blood mononuclear cells from a single sample. Normal stimulation index ranges and frequency of recognition (percentage of subjects showing stimulation index greater than or equal to 2) of each synthetic peptide are also shown in the table. Patient cells were not tested with synthetic peptide E1-4(199–217).

Figure 2 Comparative lymphoproliferative responses of patient peripheral blood mononuclear cells and synovial inflammatory infiltrates to rubella virus synthetic peptides. Freshly isolated peripheral blood mononuclear cells (hatched bars) or synovial inflammatory infiltrates (solid bars) were incubated with heat inactivated whole rubella virus (M33 strain) or synthetic peptide representing selected regions of rubella virus E1, E2, and C proteins as indicated in the table. As a result of insufficient cell numbers not all synthetic peptides were tested with synovial inflammatory infiltrates.
Lymphocyte stimulation assays performed with E2 synthetic peptides (table; fig 1, E2) showed positive responses of peripheral blood mononuclear cells to seven of 10 synthetic peptides tested. One sequence represented in peptide E2-6 (50–72) induced a particularly strong response (B1) which was seven fold higher than the highest stimulation index observed with this synthetic peptide in normal subjects. Similar response patterns to E2 synthetic peptides were observed in synovial inflammatory synovial infiltrate studies (fig 2, E2) with the exception of peptide E2-4 (35–58v), which induced proliferation of synovial inflammatory infiltrates but not peripheral blood mononuclear cells.

C Peptide studies (table; fig 1, C) also showed a high frequency of recognition (5/8) of these sequences by the patient’s peripheral blood mononuclear cells; however, the stimulation indices measured were similar to those observed with normal cells. Only three synthetic peptides were tested with synovial inflammatory infiltrates and all showed responses comparable with peripheral blood mononuclear cells (fig 2, C).

Figures 1 and 2 and the table also show synthetic peptide responses relative to those observed when peripheral blood mononuclear cells and synovial inflammatory infiltrates were stimulated with heat inactivated whole rubella virus.

Discussion
The abnormally increased cellular immunoreactivity to rubella virus antigens observed in peripheral blood mononuclear cells and synovial inflammatory infiltrates collected in this follow up study of a patient with chronic rubella associated arthritis are consistent with previous reports of increased lymphoproliferative reactivity to whole rubella virus in this patient. Consistent observations of rubella virus specific cellular hyperreactivity suggest chronic antigenaemia or low level persistence of rubella virus. Although rubella virus was detected by cocultivation techniques in synovial inflammatory infiltrates of this patient several years earlier, rubella virus genomic sequences were not detected in either peripheral blood mononuclear cells or synovial inflammatory infiltrates at this time, nor have they been detected by rubella virus-specific polymerase chain reaction in blood or synovial fluid samples collected on other occasions (unpublished data). Rubella virus has been detected by polymerase chain reaction amplification techniques in serial studies of peripheral blood mononuclear cells obtained from two other patients with rubella associated arthritis, however, but became undetectable after the start of immunosuppressive treatment (Mitchell L A, unpublished data). Hence, failure to detect rubella virus by polymerase chain reaction in this patient’s cells at this time was not surprising. Despite the sensitivity of rubella virus polymerase chain reaction detection, however, owing to the small sample sizes used, additional amplification by coculture techniques or cell activation may be necessary to further amplify the rubella virus if it is present in relatively few cells in the populations being evaluated.

By using panels of synthetic peptides representing selected non-overlapping and overlapping synthetic rubella virus E1, E2, and C proteins to stimulate the proliferation of freshly isolated peripheral blood mononuclear cells and synovial inflammatory infiltrates collected on one occasion from this patient with chronic rubella associated arthritis, we observed abnormally high levels of lymphocyte reactivity to some synthetic peptides as well as an increased frequency of recognition of synthetic peptides within each panel compared with that observed in normal healthy rubella virus immunoreactive subjects. The synthetic peptides used were originally synthesised for antibody binding studies and contain sequences predicted to be exposed on the protein surface. Hence these synthetic peptides may only partially represent the antigenic repertoire of the rubella virus proteins. Analysis of rubella virus E1, E2, and C sequences for a propensity to form amphipathic helices that have been associated with T cell antigenic sites in other proteins, however, showed that several of the rubella virus synthetic peptides contained such regions (indicated with an asterisk in the table). These synthetic peptides had been evaluated in earlier studies for their relative abilities to stimulate proliferative responses of peripheral blood mononuclear cells of normal healthy rubella virus reactive subjects. The same studies also showed that the antigenic sites represented in these synthetic peptides were also broadly recognised across several different major histocompatibility class II genotypes. Hence these relatively large synthetic peptides may represent single immunodominant antigenic sites or clusters of T cell antigenic sites. These earlier studies also showed that the synthetic peptides were recognised by rubella virus specific T cell lines, suggesting that these sequences are likely to be representative of naturally processed rubella virus peptides.

In the patient’s peripheral blood mononuclear cells strong cellular responses were observed to the synthetic peptides E1 (219–239), E1 (234–252), E1 (389–411), and E1 (462–481). The first two sequences have been associated with lymphocyte stimulatory activity and with binding of rubella virus specific haemagglutination inhibiting and neutralising antibodies in normal subjects and hence may play a part in immune protection. This patient has shown consistently low levels of antibody reactive with the E1 neutralising domain synthetic peptide (213–239) over the past seven years. Lack of correlation between cellular and antibody reactivity measured with these synthetic peptides has also been observed in studies with normal healthy subjects, however, and may reflect waning immunity. To help resolve this, studies are underway to determine T and B cell antigenic sites and to establish the kinetics of immunoreactivity to this domain.
after rubella virus natural infection and immunisation. The last two sequences, E1(389–411) and E1(462–481), were predicted to contain T cell antigenic sites on the basis of their primary structure. Otherwise their significance in the immune response to rubella virus is unknown at this time. The relatively lower reactivity observed with the synthetic peptide E1(389–412) compared with E1(389–411) may reflect the influence of flanking residues on peptide recognition by T cell receptors. Abnormally increased reactivity of patient peripheral blood mononuclear cells was also observed with the E2 synthetic peptide (50–72); however, its role in rubella virus immunity is also unknown. Although limitations of cell numbers precluded testing of synovial inflammatory infiltrates with all synthetic peptides, the response patterns of synovial inflammatory infiltrates were generally similar to those observed with the patient’s peripheral blood mononuclear cells. The stimulation indices measured with synovial inflammatory infiltrates were generally lower than with peripheral blood mononuclear cells, however, despite similar background counts in unstimulated controls. This finding was in contrast with previous observations with synovial inflammatory infiltrates of this patient using crude rubella virus antigen preparations in lymphocyte stimulation assays. This may reflect either lower numbers of synthetic peptide reactive lymphocytes or true clonal differences between the two cell populations, or technical impediments such as the release of proteases or thymidine from endstage inflammatory cells (neutrophils and macrophages), which were inevitable contaminants of this cell population and would have interfered with the uptake of the radiolabelled thymidine. The higher response of synovial inflammatory infiltrates to the synthetic peptide E1-10 (226–258) however, was notable as this sequence partially overlaps with E1(213–239), which has been shown to stimulate lymphocyte proliferation and to react with rubella virus-neutralising antibodies. Interestingly, E1-10(226–258) represents a wild type rubella virus (M33 strain) sequence; hence the relatively high response of synovial inflammatory infiltrates to this synthetic peptide may be indicative of local persistence of rubella virus in cells other than those examined here.

The data show that stimulation indices observed in stimulations with certain synthetic peptides were considerably higher than those observed with whole rubella virus. This has been occasionally observed in studies of normal subjects and may reflect preferential uptake and processing, or direct binding, of short synthetic peptides by antigen processing cells. Alternatively, the relatively high peptide response may reflect an increased frequency of responder cells for a particular sequence. Finally, it should be considered that the amino acid sequences of the synthetic peptides may share partial homology with other heteroantigens or autoantigens and thus would be capable of stimulating the proliferation of memory T cells within the peripheral blood mononuclear cell or synovial inflammatory infiltrate populations. The existence of such sequences in the polyribonucleotide has not been established at this time, however.

Observations of similar patterns of immunoreactivity of synovial inflammatory infiltrates with synthetic peptides indicate that these methods will be useful for antigenic site mapping in inflammatory cell populations. Future investigations will use cloned T cell lines prepared from peripheral blood mononuclear cell and synovial inflammatory infiltrate populations to determine the exact antigen sites lying within these immunoreactive regions of rubella virus proteins and their relative roles in the development of protective (or adverse) immunity to rubella virus.

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