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# Absence of Cytotoxic Effect of Selected Pathogens on HLA B27 Positive Fibroblasts (40310)

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There is strong suggestive evidence that at least one of the seronegative spondyloarthropathies, Reiter's disease (RD), follows genital exposure to certain infectious agents including *Chlamydia trachomatis* and *Ureaplasma urealyticum* (1). In addition, postdysenteric RD and similar forms of acute reactive arthritis are known to follow enteric infections with Enterobacteriaceae such as *Salmonella* and *Yersinia* (2). Eventually, some patients with these acute post-infective arthropathies may develop chronic sequelae identical to those seen in ankylosing spondylitis (AS) (1).

It is now firmly established that these same seronegative spondyloarthropathies are strongly associated with the B-locus histocompatibility antigen HLA B27 (3). Thus it appears that exposure to certain specific microbial agents in a genetically susceptible host may be a prerequisite for the development of this spectrum of acute-to-chronic rheumatic disease.

Several possibilities emerge from this concept. Firstly, the B27 antigen present on cell surfaces might facilitate microbial attachment and invasion. Secondly, the cell surface antigen B27 might share antigenic similarities with the microbial agents initiating these diseases. Under this circumstance the body's host defense mechanism may not recognize the agents as foreign and antigenic. Thirdly, the chromosomal locus which codes for HLA B27 is located within the major histocompatibility complex (MHC) region of the sixth autosomal chromosome. There is strong evidence in other species and suggestive evidence in man that immune response genes are also located within the same genetic complex, and that such genes may be linked to the HLA loci (4). Conceivably, immune responsiveness controlled by HLA-linked genes may be responsible for the development and/or propagation of connective tissue inflammation typified as RD and AS.

A first step in elucidating the potential role of MHC gene products in RD and AS is to explore their influence on cell surface susceptibility to implicated pathogens. Using a radiocytotoxicity assay previously standardized by cell counts and correlated to dye exclusion, cytotoxicity of implicated pathogens on human cells was investigated. The experiments reported here indicate that HLA B27 plays no such direct role in initiating the tissue lesion of the seronegative spondyloarthropathies.

*Material and methods. Target cells.* Human skin fibroblasts were cultured from 4mm full depth punch biopsies of normal and outpatient volunteers with RD or AS who had been typed for the absence or presence of HLA B27. It has been demonstrated that fibroblasts of B27-positive individuals retain their HLA surface markers for at least 12 weeks in vitro (5, 6). Explants ½mm$^3$, devoid of epidermis and subcutaneous fat, were secured in 25 cm$^2$ culture flasks (Falcon, Oxnard, CA) by surface tension of the culture medium. Cultures were established in Eagle's BME (Earle's salts) (Gibco, Grand Island, NY) supplemented with penicillin 100 units/ml, streptomycin sulfate 11 μg/ml, L-glutamine 2 m*M*, and 15% unheated fetal calf serum (FCS) (Gibco, Grand Island, NY), and kept in 5% $CO_2$ at 37°. Medium was changed weekly for 2 weeks, twice weekly thereafter. After the first change, all further culture was in the absence of antibiotics. At 4–5 weeks, the confluent fibroblast monolayers were trypsinized (0.25% in Hanks (HBSS; Gibco, Grand Island, NY), pH 8.2, 5′ at 37°) and subcultured in 1:2 splits. After four subcultures, the cells were assayed for bacterial and mycoplasmal contamination. Fibroblasts for cytotoxicity targets were harvested at late log growth phase and used only within 5th–20th generations.

*Pathogens.* The following pathogens, obtained from sources indicated, were cultured by standard methods. At least three subcul-



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tures preceded the final effector organisms used to assay cytotoxicity. Identity of later subcultures was reconfirmed by source labs.

| | | |
|---|---|---|
| *Yersinia enterocolitica* | type 8 | Dr. R. Weaver, Center for Disease Control, Atlanta, GA. |
| *Salmonella minnesota* | 595 | Dr. G. Kalmansen, Wadsworth VA, Los Angeles. |
| *Cytomegalovirus* | AD-169 | Dr. M. Fiala, Harbor General Hospital, Los Angeles; Dept. of Infectious Diseases. |
| *Herpes simplex virus* | type 2 | |
| *Ureaplasma urealyticum* | T-960 | National Institute of Allergy and Infectious Diseases, Bethesda, MD. |
| *Mycoplasma hominis* | 1001 | Dr. M. Shepard, Naval Medical Field Research Lab, Camp Lejeune, N.C. |
| *Chlamydia trachomatis* | UW-3 (type D) | Dr. S. P. Wang, Univ. of Washington; Dept. of Pathobiology. |
| *Chlamydia trachomatis* | UW-5 (type E) | |

*Cytotoxicity assay.* Fibroblast cultures were trypsinized into fresh culture medium and adjusted to $1.0 \times 10^5$ cells per ml. 1 $\mu$Ci/ml $Na_2{}^{51}CrO_4$ (ICN, Irvine, CA) was added and the suspension distributed in 1 ml aliquots to sterile flat-bottomed glass tubes (Cal Gass 15-105) which could subsequently be inserted in a well-type Nuclear Chicago $\gamma$-counter. Gas phase of each tube was equilibrated with 5% $CO_2$ in air, and the tubes were capped and incubated at 37°. At 16 hr the adherent fibroblast monolayers were washed with 1 ml/tube HBSS containing 10% FCS. After 1 more hr the wash was repeated, and the monolayers were covered with 1 ml of fresh culture medium. 0.10 ml of selected log-phase pathogen, adjusted to proper multiplicity of infection (MOI), was added to each tube. Positive controls received 6N HCl; negative controls received medium only. At intervals after the addition of pathogens, supernatants were transferred to separate $\gamma$-counter tubes, the remaining monolayers were gently rinsed with 5% FCS in HBSS, and the rinses were pooled with supernatants. Tubes with media and tubes with cells were counted for $\gamma$-emission. Fractional $^{51}Cr$ release (FR) for each cell-medium pair was expressed as

$$^{CMP}(\text{medium})/{}^{CMP}(\text{cells}) + {}^{CMP}(\text{medium})$$

and specific cytotoxicity of each pathogen as

$$^{FR}(\text{pathogen}) - {}^{FR}(\text{spontaneous})/{}^{FR}(\text{maximum}$$

Figures 1 and 2 illustrate the course of a prototype assay, used to determine suitable levels of target cell label. In Fig. 1, spontaneous, intermediate, and maximum $^{51}Cr$ release are achieved in culture medium, 70% distilled water ($dH_2O$), and 0.1 *M* sodium dodecyl sulfate (SDS) respectively. Results are expressed as fractional $^{51}Cr$ release. In Fig. 2, the $^{51}Cr$ release effected by $dH_2O$ is illustrated as specific cytotoxicity by relating it to a baseline represented by spontaneous release.

*Results.* Table I shows the mean fractional $^{51}Cr$ release of three B27-negative and three B27-positive fibroblast strains exposed to each pathogen. In Table II the specific cytotoxicity of each pathogen on these target fibroblast strains has been calculated. Over the range of dilutions used ($10^4$, $10^0$, and $10^{-4}$ MOI), paired *t* tests revealed no significant difference in the cytotoxic effect of any one pathogen on B27-positive compared to B27-negative fibroblasts with the apparent exception of *Ureaplasma urealyticum.* However, the differential killing for this organism is almost certainly not significant since it only repre-

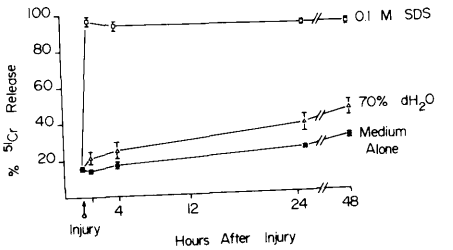


FIG. 1. Fractional $^{51}Cr$ release. Cytotoxic effect of 70% $dH_2O$ on normal human skin fibroblasts. Data points represent mean ± SEM of three determinations.

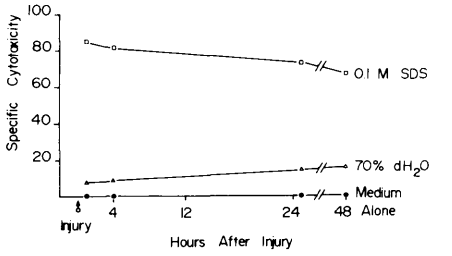


FIG. 2. Specific cytotoxicity. Results of Fig. 1 adjusted to spontaneous release baseline. Data points represent FR (X) − FR (Spontaneous)/FR (Maximum).

TABLE I. MEAN FRACTIONAL $^{51}Cr$ RELEASE OF B27- AND B27 FIBROBLASTS IN PRESENCE OF PATHOGENS.

| Viable organism | Cytotoxicity read at hours | Mean fractional $^{51}Cr$ release ± SEM[a] | | | | | |
|---|---|---|---|---|---|---|---|
| | | $10^4$ MOI | | $10^0$ MOI | | $10^{-4}$ MOI | |
| | | B27- | B27 | B27- | B27 | B27- | B27 |
| Y. enterocolitica type 8 | 24 | .626 ± .057 | .683 ± .074 | .444 ± .062 | .578 ± .092 | .411 ± .072 | .568 ± .123 |
| S. minnesota 595 | 48 | .929 ± .017 | .876 ± .004 | .913 ± .013 | .891 ± .016 | .912 ± .022 | .885 ± .019 |
| CMV AD-169 | 24 | .177 ± .030 | .180 ± .059 | .166 ± 0.29 | .119 ± .018 | .194 ± .026 | .162 ± .022 |
| HSV-2 #1000 | 24 | .162 ± .033 | .154 ± .036 | .194 ± .045 | .110 ± .005 | .234 ± .060 | .153 ± .008 |
| U. urealyticum T-960 | 18 | .213 ± .063 | .164 ± .008 | .219 ± .080 | .172 ± .009 | .211 ± .027 | .155 ± .004 |
| M. hominis #1001 | 18 | .245 ± .081 | .170 ± .004 | .183 ± .024 | .187 ± .025 | .169 ± .019 | .171 ± .002 |
| C. trachomatis UW-3 (D) | 24 | .245 ± .024 | .217 ± .013 | .246 ± .024 | .244 ± .011 | .255 ± .029 | .266[b] |
| C. trachomatis UW-5 (E) | 24 | .242 ± .030 | .246 ± .027 | .246 ± .039 | .214 ± .008 | .254 ± .036 | .258 ± .022 |

[a] Three experiments were performed.
[b] One experiment performed, due to technical difficulties.

TABLE II. SPECIFIC CYTOTOXICITY OF PATHOGENS ON B27- AND B27 FIBROBLASTS. SIGNIFICANCE.

| Viable organism | Cytotoxicity read at hours | Specific cytotoxicity | | | | | | Paired T | Significance |
|---|---|---|---|---|---|---|---|---|---|
| | | $10^4$ MOI | | $10^0$ MOI | | $10^{-4}$ MOI | | | |
| | | B27- | B27 | B27- | B27 | B27- | B27 | | |
| Y. enterocolitica type 8 | 24 | .289 | .108 | .084 | −.011 | .046 | −.023 | 1.538 | $P > .10$ |
| S. minnesota 595 | 48 | .326 | .312 | .309 | .328 | .308 | .322 | −0.583 | $P > .10$ |
| CMV AD-169 | 24 | .012 | .050 | −.001 | −.024 | .032 | .028 | −0.222 | $P > .10$ |
| HSV-2 #1000 | 24 | −.006 | −.169 | .032 | −.035 | .079 | .017 | 2.939 | $P > .025$ |
| U. urealyticum T-690 | 18 | .037 | .010 | .043 | .018 | .035 | .001 | 10.74 | $P < .001$ |
| M. hominis #1001 | 18 | .069 | .016 | .007 | .033 | −.007 | .017 | 0.038 | $P > .10$ |
| C. trachomatis UW-3 (D) | 24 | −.020 | −.008 | −.019 | .019 | −.010 | −.010 | 1.545 | $P > .10$ |
| C. trachomatis UW-5 (E) | 24 | −.023 | .021 | −.019 | −.011 | −.011 | .033 | −2.667 | $P > .05$ |

sents a difference between 1 and 2% nonspecific versus 3 and 4% specific cytotoxicity. The $t$ value is large because the two ranges were very small and did not overlap. The different pathogens damaged targets to different extents, but none truly differentiated between B27-negative and B27-positive fibroblasts.

*Discussion.* Over 90% of patients with RD or AS bear B27 on their cell surface. Conceivably, the presence of this membrane polypeptide might render the cells more vulnerable to direct attack by certain pathogens, notably those temporally incriminated with patients with RD and other post-infective arthropathies. Such susceptibility would permit rapid attachment, invasion, and cell destruction before host immune recognition and defense were fully mobilized. However, our study shows that B27-positive skin fibroblasts do not differ from B27-negative skin fibroblasts with respect to their susceptibility to damage by selected pathogens.

Other relevant organisms assayed in other *in vitro* systems may possibly demonstrate an enhanced and differential cytotoxicity toward B27-positive target cells, but this would appear to be unlikely.

*Summary.* A sensitive index of *in vitro* cell damage has been used to investigate the possibility that HLA B27-positive fibroblasts are peculiarly susceptible to those infectious agents incriminated in the seronegative spondyloarthropathies. No evidence for differential susceptibility related to the presence or absence of the B27 antigen could be demonstrated.

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