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Interaction of Influenza A Virus with Human Peripheral Blood Lymphocytes

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Peripheral blood lymphocytes cultured in the presence of phytohemagglutinin, concanavalin A, or pokeweed mitogen were exposed in vitro to influenza A virus. The synthesis of several virus-specific proteins, including the nucleoprotein, membrane protein, and nonstructural 1 protein were detected, although no infectious virus was produced by the lymphocyte cultures. Evidence was obtained that only a subpopulation of mitogen-transformed cells would support virus protein synthesis. A comparison of the interactions of influenza A virus with lymphocytes from normal individuals and from rheumatoid arthritis patients showed that the same range of virus-specific proteins were made, in similar quantities, regardless of the source of lymphocytes.

Previous studies on the interactions of influenza viruses with human and mouse lymphoid cells in vitro have shown that although both B and T lymphocytes have surface receptors for a variety of influenza virus strains (29), incubation of influenza virus with lymphocytes in vitro results in rapid inactivation of the virus (31). It has been suggested (16) that virus inactivation might not simply result from virus attachment to the lymphocyte surface, since electron microscopic studies demonstrated that virus particles are taken up into cytoplasmic vacuoles. However, the subsequent fate of the virus within the lymphocyte, apart from the eventual inactivation of its infectivity, has not been elucidated further.

We have been interested in establishing whether any virus replication events occur after uptake of the virus into the lymphocyte. Synthesis of virus-specific proteins within the lymphocyte could influence the course of cell-mediated immune responses to influenza infection. In addition, we have investigated the interaction of influenza virus with peripheral blood lymphocytes (PBL) from rheumatoid arthritis (RA) patients compared with influenza virus interaction with PBL isolated from normal individuals. One hypothesis advanced for the etiology of RA has been that a virus infection might result in an alteration in function of effector cells of the immune system causing derepression of cell function, selective proliferation of specific cell clones, or loss of regulation of B cell functions or a combination of these effects (11; B. P. Marmion, *Infective Agents and the Immunopathology of Rheumatoid Arthritis, Clinics in Rheumatic Diseases*, in press). Using influenza virus, which exhibits an absolute dependence on host cell nuclear function during its replication (2, 15, 18, 21, 22, 24), it was hoped that differences between PBL from RA patients and those from normal individuals might be revealed in the types of interactions exhibited with influenza virus.

The present study demonstrates that influenza virus inactivation by human PBL is preceded by an incomplete cycle of virus replication in which several virus-specific proteins are synthesized. No significant differences were detected between the number and amounts of virus-specific proteins synthesized by PBL isolated from RA and the number and amounts of those synthesized by PBL from normal individuals.

MATERIALS AND METHODS

Preparation of PBL cultures. For preparation of PBL cultures, 10 ml of venous blood was collected in 0.15 ml of preservative-free heparin (1,000 IU/ml.; Paines and Byrne, Ltd., Greenford, England), and the lymphocytes were separated and cultured as previously described (7). PBL cultures were transformed by addition of 30 μg of phytohemagglutinin (PHA) (Wellcome Reagents, London, England) per ml.

Growth of virus stocks. The various influenza A viruses used were grown in fertile hens' eggs 11 days old and assayed by hemagglutination as described by Borland and Mahy (4). Aliquots of 2 ml of the infected allantoic fluid were stored at −70°C. Infections with the A/England/42/72 strain of influenza virus were carried out with a frozen stock of virus

which had a hemagglutination titer of 128 hemagglutination units per ml and an infectivity of 3.0×10^7 50% egg infective doses.

Infection of CEF and PBL cultures with influenza virus. Confluent chicken embryo fibroblast (CEF) monolayers on 3-cm plastic petri dishes containing approximately 3×10^6 cells were prepared and infected at a multiplicity of 10 to 20 plaque-forming units per cell as described by Borland and Mahy (4). PBL to be infected were collected by centrifugation, suspended in phosphate-buffered saline (PBS), recentrifuged, and then suspended in 1 ml of infected allantoic fluid diluted to provide a multiplicity of 10 to 20 plaque-forming units per cell. The cultures were rotated gently for 60 min at room temperature during adsorption. After virus adsorption, the inoculum was removed from the cells, the cultures were washed once with PBS, and then PBL were suspended in 1 ml of medium (1×10^6 to 2×10^6 cells per ml).

Labeling of cultures with [^{35}S]methionine. [^{35}S]methionine labeling of cultures was carried out as previously described (21).

Polyacrylamide gel electrophoresis and autoradiography. The procedures for the analysis of [^{35}S]methionine-labeled polypeptides by discontinuous polyacrylamide gel electrophoresis and autoradiography were as previously described (17).

Indirect immunofluorescence assay. Samples containing approximately 2×10^5 cells were removed from PBL cultures and centrifuged onto clean microscope slides, fixed in ice-cold acetone for 5 min, and allowed to air-dry; the samples were stored at 4°C in the presence of anhydrous calcium chloride until they could be assayed.

Samples were incubated for 60 min at 37°C with rabbit antiserum raised against the ribonucleoprotein antigen of influenza A virus grown in eggs (fowl plague virus, Rostock strain); the antiserum, kindly donated by D. N. Planterose, was appropriately diluted in PBS. The samples were then gently but extensively washed for 30 min in three changes of PBS and subsequently incubated for 60 min at 37°C with fluorescein-conjugated sheep antirabbit serum (Wellcome Reagents) diluted 1:7 with PBS. The samples were then washed for 30 min in three changes of PBS. Finally, the samples were mounted in glycerol-PBS medium (9:1) and analyzed under ultraviolet light on a Zeiss microscope. Photomicrographs were taken on AGFA 50L film.

Measurement of SR. The stimulation ratio (SR) after exposure to virus or to the mitogen PHA was measured in duplicate cultures of PBL containing 1×10^6 to 2×10^6 cells in plastic tubes by incubation at 37°C for 24 h with 5 μCi of [^{3}H]thymidine (20 to 30 Ci/mmol; Radiochemical Centre, Amersham, England) per ml in RPMI medium containing 2% heat-inactivated fetal calf serum. At the end of the labeling period, cells were washed with ice-cold saline and suspended in saline containing 0.1% sodium dodecyl sulfate and 100 μg of calf thymus deoxyribonucleic acid as carrier. An equal volume of 1 M perchloric acid was added to precipitate cellular material; the samples were collected onto Whatman GF/C filters, washed with 8% trichloroacetate and absolute alcohol; and acid-insoluble radioactivity was determined by scintillation counting in toluene-based scintillant. The SR was determined as the ratio of [^{3}H]thymidine counts per minute incorporated by PHA-stimulated or virus-infected PBL cultures compared with the counts per minute incorporated into cultures which received medium containing neither PHA nor virus.

Analysis of deoxyribonucleic acid synthesis by autoradiography. Five replicate PBL cultures in plastic tubes containing 10^6 cells were labeled for 60 min at 37°C with 5 μCi of [^{3}H]thymidine per ml. Duplicate samples of 2×10^5 cells were removed from each culture and centrifuged onto clean microscope slides and then fixed for a total of 30 min in two changes of methanol-acetic acid (9:1) at 4°C. The samples were rinsed in methanol, allowed to air-dry, and subsequently processed for autoradiography. Slides were coated with K2 emulsion (Ilford, Moberly, Cheshire, England) and allowed to expose at 4°C for 3 weeks, and autoradiographs were then developed and stained with Giemsa stain.

Lymphoblastoid cell line RPMI-1788. Lymphoblastoid RPMI-1788 cells were obtained from Searle Diagnostic (High Wycombe, Bucks, England) and were normally cultured in RPMI-1640 (Gibco Biocult, Glasgow, Scotland) medium supplemented with 20% fetal calf serum; 2 mM glutamine; nonessential amino acids; the antiobiotics penicillin (100 μg/ml), streptomycin (100 μg/ml), kanamycin (100 μg/ml), and amphotericin B 3 μg/ml; and sodium bicarbonate.

RESULTS

Synthesis of virus-specific polypeptides in PBL cultures exposed to influenza virus. In a preliminary experiment, cells of a lymphoblastoid cell line (RPMI-1788), derived originally from the peripheral blood of a normal person, were infected with several different influenza A virus strains. The virus strains used were an avian influenza A virus Dobson strain which had been adapted to undergo productive replication in mammalian cells (19); two human influenza A virus strains, PR8 and WS, both of the H0N1 type; and an H3N2 isolate (England/42/72). The infected RPMI-1788 cell cultures and a set of confluent CEF monolayers infected with the same viruses were labeled with [^{35}S]-methionine (5 μCi/ml) between 3 and 4 h post-infection. The proteins synthesized during this period were analyzed by polyacrylamide gel electrophoresis and autoradiography (Fig. 1).

Virus-specific proteins were synthesized in RPMI-1788 cells exposed to the virus strains PR8 and England/42/72. The virus protein synthesized in the largest amount was the nucleoprotein (NP), with appreciable amounts of the nonstructural protein 1 (NS_1) and trace amounts of the membrane (M) protein being made in the cultures infected with England/42/72.

A subsequent experiment was carried out with RPMI-1788 cells and PBL prepared from the

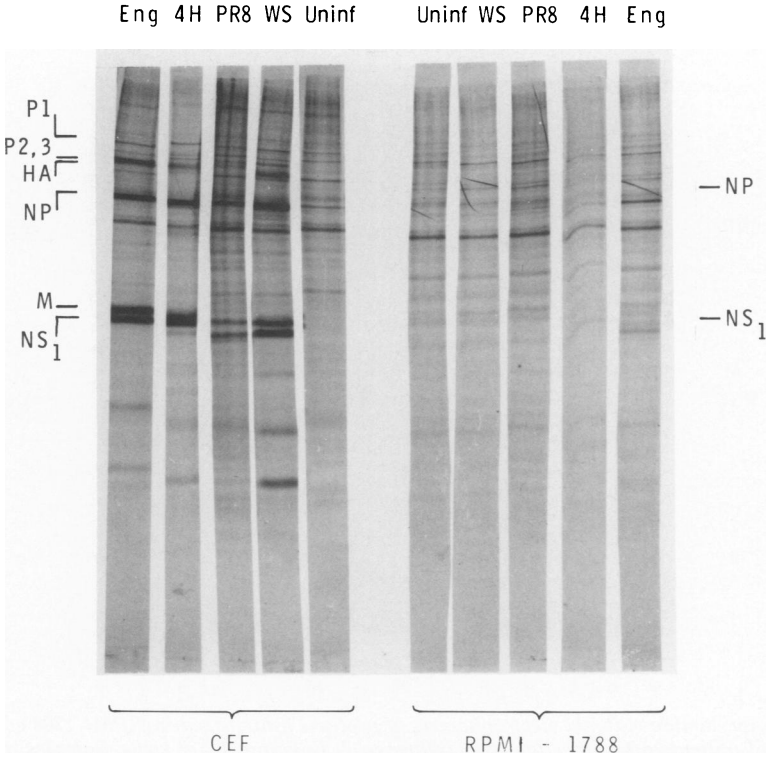

FIG. 1. *Polyacrylamide slab gel electrophoresis of proteins synthesized in CEF or RPMI-1788 cells after infection with the influenza A viruses England/42/72 (Eng), Dobson 4H, PR8, or WS. Cells were infected and then labeled with 5 μCi of [^{35}S]methionine per ml between 3 and 4 h postinfection. The cells were harvested, suspended in Laemmli buffer, and electrophoresed on a 17.5% polyacrylamide gel. The newly synthesized proteins were detected by autoradiography. Labeling of virus-specific polypeptides refers to the proteins synthesized in cells infected with the Eng strain of influenza virus. Abbreviations: Uninf, uninfected; P_1, P_2, P_3, polymerase-associated proteins; HA, hemagglutinin.*

peripheral blood of RA or control (osteoarthritis) patients. The PBL cultures were stimulated with 30 μg of PHA per ml and infected with England/42/72 virus on day 5 after mitogen stimulation. PBL cultures which had not been transformed with PHA did not support the synthesis of any virus protein. It was of interest to establish if longer exposure to virus resulted in the synthesis of additional virus proteins. Cultures were infected with England/42/72 virus and subsequently labeled with [^{35}S]methionine from 0 to 6 or from 0 to 24 h (Fig. 2). The shorter labeling period showed the synthesis of the virus-specific proteins NP and NS_1; however, on prolonged labeling additional polypeptides were noted which migrated in positions just in front of the NP band and in the M position. Various amounts of M protein seemed to be synthesized in the three cell types, a much greater amount being synthesized in the PBL isolated from the RA patient. In addition, synthesis of a protein migrating in the position of the uncleaved hemagglutinin polypeptide was seen in some samples. Partial peptide digests (10) of the NP and NS_1 synthesized in virus-infected PBL were compared with those of proteins synthesized in CEF. Polypeptides from infected PBL which migrated with mobilities similar to those of polypeptides from infected CEF had similar digest patterns, confirming their virus-specific origin. The medium from infected PBL cultures was analyzed with an egg infectivity assay. By 6 h postinfection, only 5% remained of the total virus infectivity present in the inoculum, and 77% of the infectivity that remained was present within the cells. No evidence was obtained for productive replication of influenza virus in these PBL cultures. These findings are in agreement with the observations on inactivation of influenza virus by lymphocytes described by Zisman and Denman (31) and Denman and Pinder (13).

Population of PBL involved in interaction with influenza virus. We were interested to establish what proportion of the total PBL

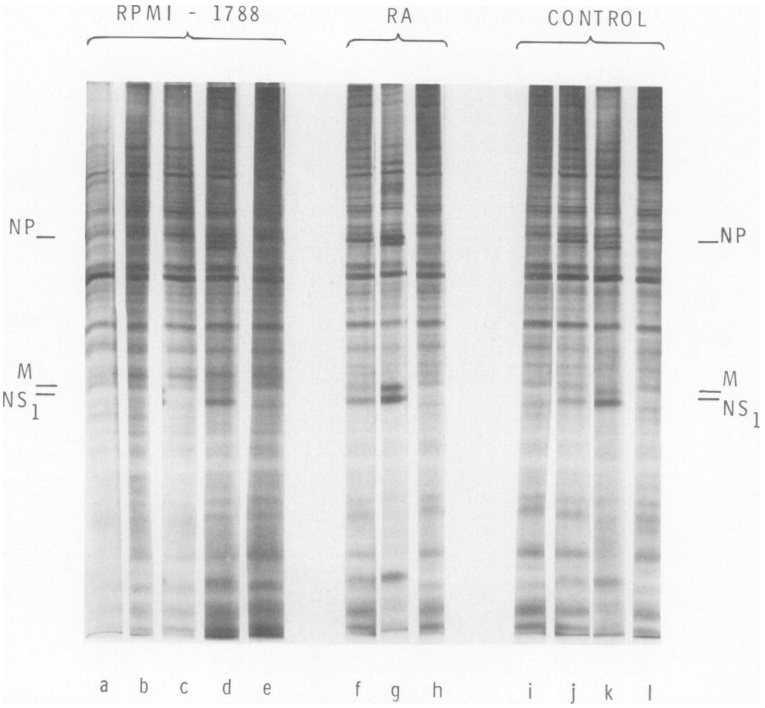

FIG. 2. *Polyacrylamide slab gel electrophoresis of proteins synthesized in RPMI-1788 cells and PB from RA and control patients after infection with influenza A England/42/72 virus. Samples a through d were RPMI-1788 cells infected with the virus and labeled with 5 μCi of [^{35}S]methionine per ml between 0 and 2 h postinfection (p.i.) (a), 0 and 4 h p.i. (b), 0 and 6 h p.i. (c), and 0 and 24 h p.i. (d). Sample e was uninfected RPMI-1788 cells labeled for 6 h. Samples f through h were RA lymphocytes infected with virus and labeled between 0 and 6 h p.i. (f) or between 0 and 24 h p.i. (g) or uninfected and labeled for 6 h (h). Samples i through l were PBL from control patients infected with virus and labeled between 0 and 4 h p.i. (i), 0 and 6 h p.i. (j), or 0 and 24 h p.i. (k) or uninfected and labeled for 6 h (l). Samples were run on a 17.5% polyacrylamide slab gel. Proteins are labeled as in Fig. 1.*

population was able to support virus-specific protein synthesis. PBL were prepared from RA and control patients and cultured in the presence of PHA. On day 4 after PHA stimulation the cells were exposed to influenza virus (England/42/72), and at 0, 2, 4, and 6 h postinfection, duplicate samples of individual cultures (2×10^5 cells) were harvested; subsequently, the preparations were stained, using an indirect immunofluorescence technique, with antiserum raised against influenza A virus ribonucleoprotein antigen (see Materials and Methods). Fluorescence was intense in the 6-h samples (Fig. 3) and was observed in the nucleus or the cytoplasm or both of some cells. A survey of the total population in different infected PBL samples showed that from 8 to 15% (mean, 12%) of the total number of cells exhibited strong fluorescence by 6 h postinfection. There appeared to be no significant difference in the number of cells showing fluorescence and the intensity and distribution of that fluorescence between RA and control PBL samples.

Virus infection of PBL not exposed to mitogen resulted in no detectable virus-specific protein synthesis, and labeling of nontransformed infected PBL cultures with [^{3}H]thymidine showed that influenza A virus England/42/72 (H3N2 subtype) did not itself possess mitogenic activity. This agrees with the demonstration by Butchko et al. (8) that H2N2 strains of influenza virus were mitogenic for human PBL and BALB/c mouse spleen cells, whereas A/England/42/72 was not. In the present study, PBL prepared from RA patients and controls and subsequently cultured for 24 h with A/England/42/72 virus and [^{3}H]thymidine showed SRs of 1.4 and 1.2, respectively. The magnitude of SR obtained with PHA varied considerably from sample to sample; for example, for five samples each from different control and RA patients, the SRs on day 4 after PHA stimulation varied from 2.4 to 180 and 5.6 to 162, respectively. PBL from RA or control patients did not differ significantly in this respect.

To ascertain the proportion of the total PBL

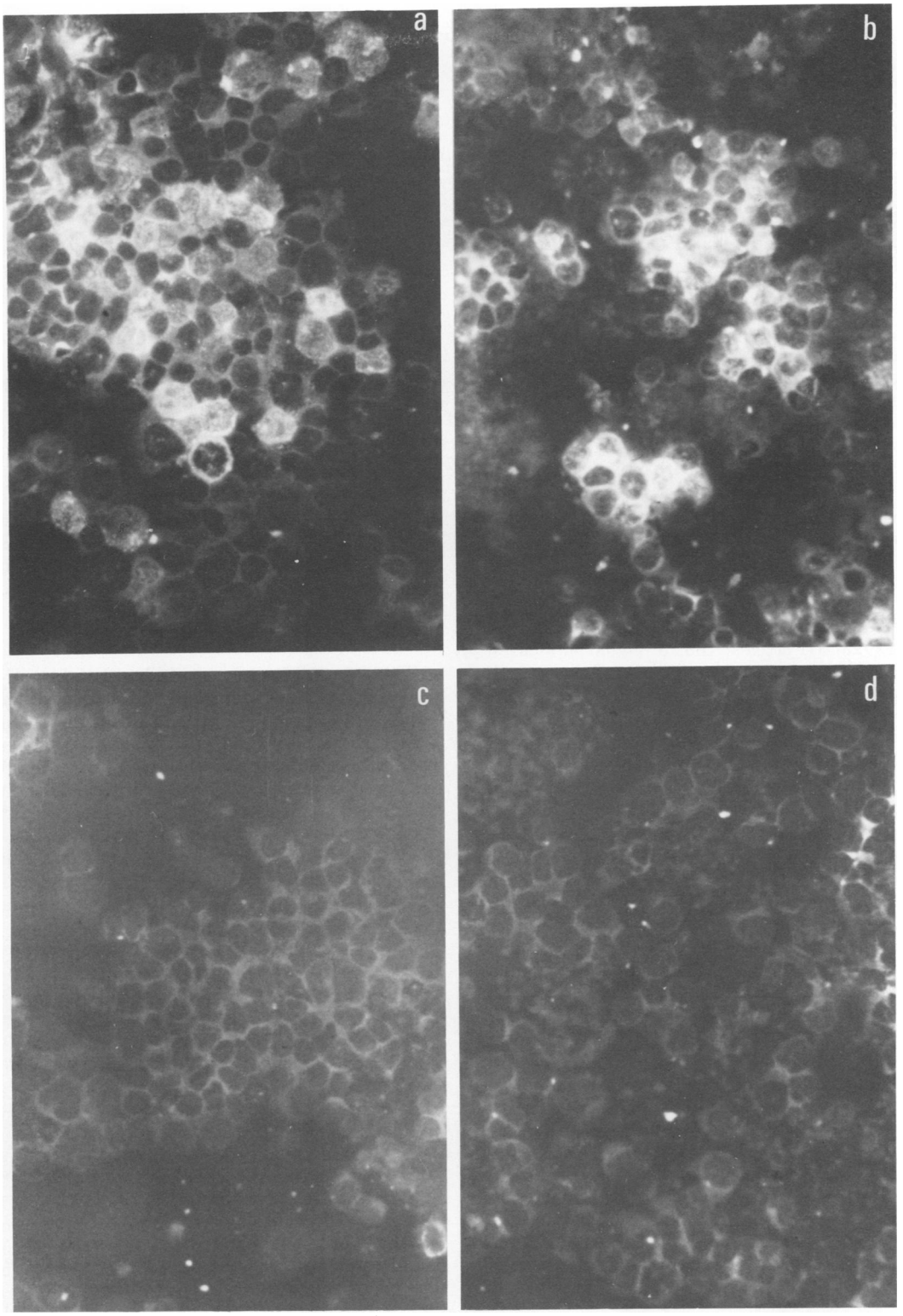

FIG. 3. *Indirect immunofluorescence staining, using anti-ribonucleoprotein serum, of influenza virus-infected PBL at 6 h postinfection. (a) Virus-infected PBL from RA patient; (b) virus-infected PBL from control patient; (c) uninfected PBL from RA patient; (d) uninfected PBL from control patient.*

population in a culture which was synthesizing deoxyribonucleic acid by day 4 after PHA stimulation, PBL cultures from control or RA patients were labeled with [^{3}H]thymidine and subsequently processed for autoradiography. The grain-positive cells were counted and expressed as a percentage of the total cell population. A total of 25% of the cells in the control samples and 21% of the cells in the RA cultures were found to be labeled with radioactive grains and thus were assumed to be synthesizing deoxyribonucleic acid at the time of harvest. Since immunofluorescence had revealed that, on average, 12% of the total cell population were able to support virus-specific protein synthesis after exposure to influenza virus, the results of the autoradiography experiment suggest that probably only a subpopulation of PHA-transformed cells was involved in virus replication events. The same virus-specific proteins were synthesized in similar amounts after influenza virus infection of PBL cultures which had been cultured in the presence of concanavalin A or pokeweed mitogen or if the cultures were depleted, using carbonyl iron, of macrophages. These observations and others in the literature (12, 29, 31) suggest that a T-cell population is involved in the interaction of influenza A England/42/72 virus (an H3N2 subtype) with PBL. Further experiments with T- and B-cell-enriched populations are necessary to investigate the precise lymphocyte population involved in the interaction and inactivation of influenza virus.

Comparison of virus-specific protein synthesis after exposure of PBL from control or RA patients to influenza virus. It had been noted in earlier experiments when virus-specific protein synthesis was followed in PBL exposed to influenza virus that more M appeared to be synthesized in samples prepared from RA compared with that in samples from control patients. It was therefore important to survey a larger number of samples to establish whether the ability to synthesize M protein was characteristic of RA PBL cultures. PBL were isolated from 23 RA patients, 9 osteoarthritic patients, and 8 normal, healthy individuals. The cells were cultured in vitro in the presence of PHA, and on day 3 after mitogen stimulation the cells were exposed to influenza A virus England/42/72. After virus adsorption, the cells were cultured for 24 h in the presence of [^{35}S]methionine (5 μCi); the samples were subsequently analyzed by polyacrylamide gel electrophoresis and autoradiography. The amounts of NP and M protein which were synthesized in the infected cultures were quantitated by making densitometer traces of the autoradiographs and weighing the appropriate peaks on the traces. A quantitative comparison was made of the amount of M protein synthesized in relation to NP protein in each sample.

In a normal productive infection with England/42/72 virus in CEF the ratio of M protein to NP synthesized in such cells was from 0.305 to 0.326, and in an abortive infection, such as that of the same virus in mouse L cells, the ratio was 0.286. The results showed that in all PBL samples in which M protein was synthesized, the amount made relative to NP was less than that characteristic of a productive infection. There seemed to be little difference in the proportions of samples able to synthesize M protein in each class of individual, and there appeared to be only small variations in the distribution of these samples into classes on the basis of the amount of M protein synthesized. These results therefore suggest that the ability of PBL to synthesize influenza virus M protein is not restricted to samples prepared from RA patients, and thus this property could not be used, as had been hoped, as an indicator of altered lymphocyte function in the RA disease state.

DISCUSSION

These experiments were designed to investigate the interaction between influenza virus and PHA-transformed and nontransformed human PBL in vitro. The experiments showed that several virus-specific proteins, NP, M protein, and NS_1, and in some samples the hemagglutinin protein, were synthesized in PHA-transformed PBL cultures exposed to influenza A virus England/42/72. These proteins were shown to be virus induced by comparison with uninfected samples run in parallel with the infected samples and on the basis of their mobilities in polyacrylamide gels compared with those from samples prepared from infected CEF cells. In addition, NP and NS_1 gave partial peptide digest patterns similar to those of proteins of the same electrophoretic mobilities which had been isolated from infected CEF cells, indicating that they were virus-coded proteins.

Previous studies have shown that rat thoracic duct lymphocytes and bone marrow cells have specific receptors for a variety of influenza A virus strains (29), that influenza virus undergoes rapid inactivation on interaction with lymphocytes in vitro, and that the virus is in fact taken up in vacuoles by these cells (16, 31). The experimental evidence presented here shows that the virus undergoes an incomplete cycle of replication within PBL similar to that described in other mammalian cells (5, 25). Further experiments are necessary to define more precisely the range of virus-specific proteins made in PBL

exposed to influenza virus; a high host background level of protein synthesis made identification of some virus proteins difficult, for example, the P and hemagglutinin proteins. Immunoprecipitation with antiserum raised against the England strain of influenza virus might allow recognition of these virus proteins if they are synthesized in PBL.

The demonstration of virus-specific protein synthesis within PBL exposed in vitro to influenza virus may have important implications in the understanding of the outcome of influenza virus infection in vivo. Although further experiments are needed to define precisely the subpopulation of PBL which interacts with influenza virus in vitro, it is likely (see Results) that a T-cell population is responsible for the virus-specific protein synthesis seen here. In line with general theories on the participation of T lymphocytes in viral infection (1, 27), it is thought that lymphocytes in influenza are involved first in the production and release of interferon, which may protect other virus-susceptible host cells (20); second in the role of helper cells in the differentiation of B-cell clones which will synthesize antibody directed against the HA antigen (26); and third as cytotoxic T cells in the killing of host cells which express virus-coded antigens on their cell surfaces (3, 6, 9, 14, 30, 32). Additional mechanisms for lymphocyte involvement in the limitation of and recovery from virus infection have included the concept that by virtue of their ability to infiltrate tissues, at susceptible sites or organs, lymphocytes may limit virulence by having specific receptors for virus, permitting virus entry but supporting an incomplete cycle of virus replication. This might effectively reduce viral infectivity in areas of highly susceptible cells. Although the experiments described here on virus-specific protein synthesis in PBL were carried out in vitro, there is at least one report (28) describing the presence of virus-specific antigens on the surface of circulating blood lymphocytes of patients suffering from influenza. Experiments are now in progress to determine whether any of the virus-specific protein synthesized in PBL exposed in vitro to influenza virus can be detected on the surfaces of PBL.

The second series of experiments was designed to test the possibility that defects in PBL cell function which might be present in RA PBL could be detected by using the synthesis of M protein in PBL exposed to influenza virus as an indicator of host cell function. Preliminary results suggested that the M protein might be synthesized in greater amounts in PBL and RA patients compared with PBL from control individuals. Such an observation was potentially interesting, since the expression of M protein on the infected cell surface is thought to be involved in the cytotoxic response to influenza virus infection, the M protein being the virus antigen responsible for the generation of cross-reactive cytotoxic T cells (3, 6). Changes in the extent of synthesis of a particular protein which is involved in cytotoxic responses to disease might be important to an understanding of possible aberrations in T-cell function in RA. However, the survey of RA samples undertaken did not reveal a specific correlation of increased M protein synthesis with PBL from RA patients. Further analysis of the interaction of influenza virus with lymphocytes is continuing with samples prepared from the synovial fluid of RA patients, since data in the literature (23) suggest that perturbations in the relative proportions of T and B cells and their respective functions are more consistently detected in lymphocytes from synovial fluid compared with PBL from the same patient.

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