MOLECULAR EVENTS IN LYMPHOCYTE DIFFERENTIATION: STIMULATION OF NONHISTONE NUCLEAR PROTEIN SYNTHESIS IN RABBIT PERIPHERAL BLOOD LYMPHOCYTES BY ANTI-IMMUNOGLOBULIN

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SUMMARY: Using a sensitive double-labelling technique, changes in protein synthesis were investigated in rabbit peripheral blood lymphocytes at early times following stimulation with heterologous anti-immunoglobulin serum (GARG) or concanavalin A (Con A). At four hours following stimulation with either GARG or Con A, a striking increase in the synthesis of a nonhistone nuclear protein of apparent molecular weight 30-40,000 was observed. These results suggest that lymphocyte activation by both anti-immunoglobulin and Con A includes as an early event synthesis of a nonhistone nuclear protein.

INTRODUCTION

The binding to surface immunoglobulin (Ig) molecules on rabbit peripheral blood lymphocytes (PBL) by anti-Ig sera is followed by blastogenic transformation of up to 90% of the cells (1-3). The high percentage of responding cells, in contrast to the low levels obtained with antigen, allows the investigation of events following interaction of ligand with antigen receptor Ig (4).

Mitogen stimulation of lymphocytes from several species initiates rapid changes in several parameters, one of the earliest being the synthesis of nonhistone nuclear proteins (5,6), which have been implicated in the regulation of DNA transcription (7). In this paper, we will report quantitative changes in nonhistone protein synthesis within four hours of anti-Ig stimulation of rabbit PBL.

MATERIALS AND METHODS

Cell preparation and culture. PBL were prepared under sterile conditions at room temperature from the blood of adult outbred rabbits from the colony at the Walter and Eliza Hall Institute of Medical Research. Buffy coat cells were separated from whole blood in heparin (10^5 units/liter) and re-suspended in RPMI 1640 (Grand Island Biological Co., San Francisco, CA)

containing sodium bicarbonate (3.4g/liter), N-2-hydroxyethylpiperazine-N'-2' ethanesulfonic acid (HEPES, 10 mM), 1% fetal calf serum (Commonwealth Serum Labs., Melbourne), 2-mercaptoethanol (5×10^{-5}M), and antibiotics (100 mg/liter streptomycin sulfate and 10^5 units/liter penicillin). Cells were cultured in RPMI 1640 in 1 ml aliquots, 2×10^6 cells/ml, in 17 x 100 mm plastic culture tubes (Type 2001, Falcon, Oxnard, CA), in a 10% CO_2-90% air atmosphere at 37°C. DNA synthesis was measured by incorporation of ^{125}IdU, 0.1 μCi/culture (100 μCi/μg, Amersham, Buckinghamshire, England), during hours 24-48 of culture. Following three washes in PBS, radioactivity was counted in a Packard Autogamma scintillation spectrometer. Since similar results were obtained with total and trichloroacetic acid-precipitable radioactivity, precipitation was routinely omitted.

Protein synthesis and preparation of nuclei. PBL ($0.5 - 1 \times 10^7$/ml) were cultured in Dulbecco's modified Eagle's Medium minus leucine and methionine (Commonwealth Serum Labs.), supplemented with glutamine (4mM), methionine (10^{-4}M), and with bicarbonate and antibiotics as for RPMI 1640. ^{3}H-leucine (50-100 μCi of 46 Ci/mmol, Amersham), or ^{14}C-Leucine (5-10 μCi of > 270mCi/mmol, Amersham) was added at the start of culture immediately following mitogen addition. At the end of the leucine pulse, the cells were centrifuged and the pellet was incubated for 15 min at room temperature with 0.5 - 1 ml 0.1% Nonidet P40 in PBS pH 7.3. The extract was layered over 0.32M sucrose in PBS containing 0.1% Nonidet P40 and centrifuged at 900 g for 7 min. The pellet, which by phase microscopy was seen to contain intact nuclei without visible cytoplasmic contamination, was dissolved in tris buffer containing sodium dodecylsulfate (SDS) and 2-mercaptoethanol, incubated for 3 min at 100°C, and analysed by SDS polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (8). All gels contained 10% acrylamide unless otherwise stated. The gels were cut into 2 mm slices, solubilized overnight at room temperature in 0.5 ml Soluene 350 (Packard Instrument Co., Downers Grove, ILL), and counted in a toluene-based scintillation fluid containing 4g PPO (Packard) and 0.30g POPOP(Packard)/liter in a Packard Tri-Carb liquid scintillation spectrometer.

Mitogens and serological reagents. Goat antibody to rabbit gamma globulin (GARG) was purchased from Commonwealth Serum Labs, and Concanavalin A from Calbiochem, San Diego, CA. Rabbit anti-allotype serum, specific for the immunoglobulin light chain b4 allotype, was the generous gift of Dr. R. Raison, Monash University, Melbourne. Rabbit IgG was isolated from the serum of immunized rabbits by zone electrophoresis and gel filtration on Sephadex G-200. It was coupled to Sepharose 4B to a ratio of 5mg IgG/ml Sepharose by the CNBr method (9).

RESULTS

Stimulation of ^{125}IdU incorporation by GARG. Sell et al (1,2) have described the kinetics and specificity of the blastogenic transformation of rabbit PBL by antisera directed against Ig allotypic determinants. In our experiments the commercially prepared GARG gave optimal stimulation at a dilution of 1:100 - 1:200, with a mean stimulation ratio (radioactivity in experimental divided by radioactivity in saline control) in 13 rabbits of 4.9 (range 1.4 - 8.8). In three experiments, the stimulatory effect of GARG on ^{125}IdU incorporation

was greatly reduced or eliminated by passing the GARG over a column of sepharose conjugated with rabbit IgG; unconjugated sepharose did not reduce the mitogenicity of the GARG. Normal goat serum did not stimulate DNA synthesis. An antiallotype serum with specificity for an Ig light chain determinant (b4) also gave significant stimulation in two rabbits of b4/b4 allotype. These results strongly suggest that the mitogenic element in the GARG is antibody to rabbit Ig. Cultures were routinely stimulated with 1:200 GARG or 5 μg/ml Con A. On a cell-for-cell basis, cultures enriched for lymphocytes by isopaque-ficoll separation gave about three-fold higher stimulation ratios for GARG than non-enriched cultures.

Nuclear protein synthesis: double label experiments. For increased sensitivity in the detection of protein synthetic variations soon after GARG stimulation, double isotope experiments were performed. After 4 hr mitogen stimulation and leucine pulse, the GARG-stimulated culture (which received ^{3}H-leucine) and the PBS control culture (which received ^{14}C-leucine) were mixed, and Nonidet P40 extraction, nuclear separation, and SDS PAGE were performed as described in Materials and Methods. The ratio of ^{3}H (stimulated)/^{14}C (control) radioactivity was calculated and graphed vs relative mobility. The same assay was performed for duplicate PBS-control cultures pulsed with ^{3}H and ^{14}C. An increase in the ^{3}H/^{14}C ratio in the GARG/PBS pair relative to the PBS/PBS pair would indicate increased protein synthesis in response to GARG, while a decrease in the ratio would signal a decrease in synthesis of proteins of a given PAGE mobility. The results of several experiments are shown in Figures 1-2. Figure 1 shows the data from a single experiment; each section of the figure represents one gel. The radioactive counts for the two isotopes were compared by normalizing them to the peak value at approximately 0.5 relative mobility; the numerical value for this point is given in the figure legends. It can be seen that qualitatively the protein profiles for control and stimulated cultures on SDS PAGE are very similar. Both GARG and Con A stimulation resulted in an overall small increase in the ^{3}H/^{14}C ratio and a striking increase at 0.6 - 0.65 relative mobility, corresponding to an apparent molecular weight of 30-40,000. When the nuclear material from GARG-stimulated cells was analyzed on 14% gels, permitting observation of nuclear histone proteins (Fig. 2), it was seen that histone protein synthesis did occur during the culture period (relative mobility 0.75 - 0.85). However,

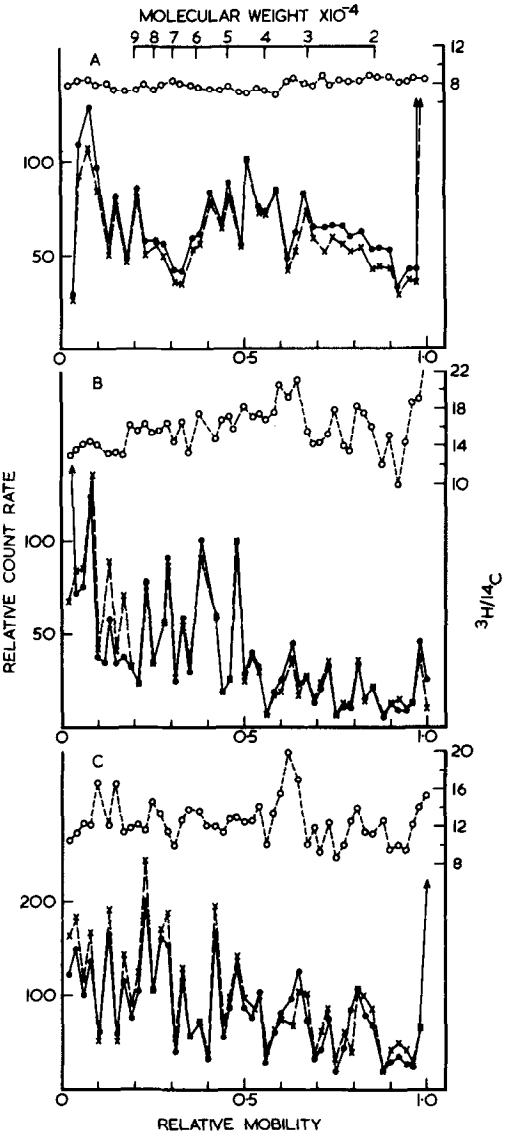

Figure 1. SDS PAGE of nuclear proteins prepared from aliquot of the cultures represented in Fig. 2. A. X--X PBS + ^{14}C-leu; 100% relative count rate = 1809 cpm. ●—● PBS + ^{3}H-leu; 100% = 12,994 cpm. O···O ^{3}H/^{14}C. B. X—X PBS + ^{14}C-leu; 100% = 737 cpm. ●—● GARG + ^{3}H-leu; 100% = 11,708 cpm. O···O ^{3}H/^{14}C. C. X--X PBS + ^{14}C-leu; 100% = 214 cpm. ●—● Con A + ^{3}H-leu; 100% 3021 cpm. O···O ^{3}H/^{14}C.

the amount of histone synthesis in the stimulated culture was not markedly increased compared to the control culture. The same was true for Con A stimulated cultures. Similar results were obtained using chromatin pre-

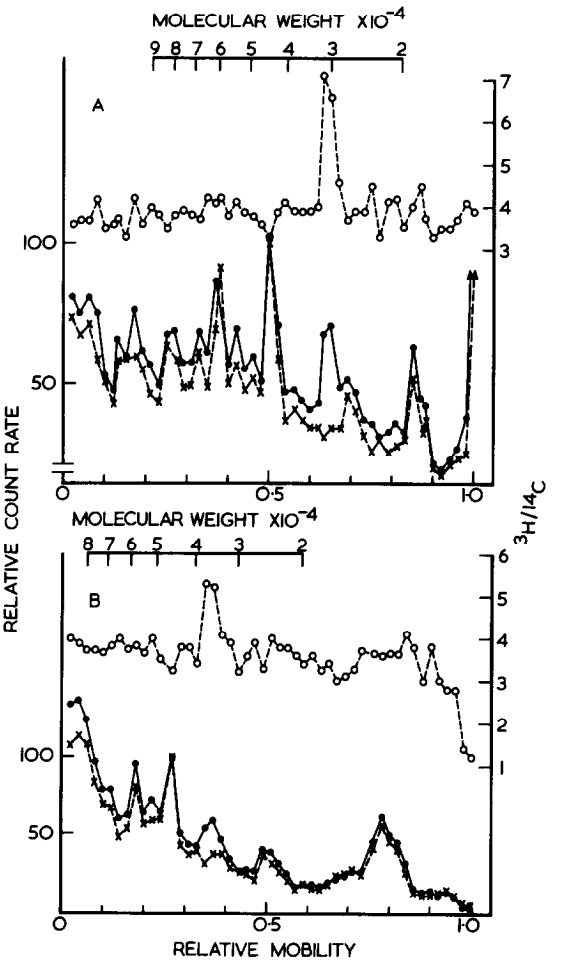

Figure 2. SDS PAGE of nuclear proteins from PBL stimulated for 4 hr with GARG or PBS. X– – X PBS + ^{14}C-leu ●—● GARG + ^{3}H-leu. O- - - O ^{3}H/^{14}C. A. 10% gel, reducing conditions, 100% relative count rate = 1016 cpm for ^{14}C, 3356 cpm for ^{3}H. B. 14% gel, reducing conditions. In other experiments, histone standards ran with and slightly ahead of the protein band seen at 0.75 - 0.85 relative mobility in this experiment, 100% relative count rate = 1157 cpm for ^{14}C, 3734 cpm for ^{3}H.

pared from isolated nuclei by tris-HCl extraction according to Huang and Huang (10). Reversing the isotopes (i.e. adding ^{3}H to the PBS control culture and ^{14}C to the mitogen-stimulated culture) did not alter the findings (data not shown).

To confirm that the protein synthetic changes induced by GARG and Con A involved molecules with identical mobilities on SDS polyacrylamide

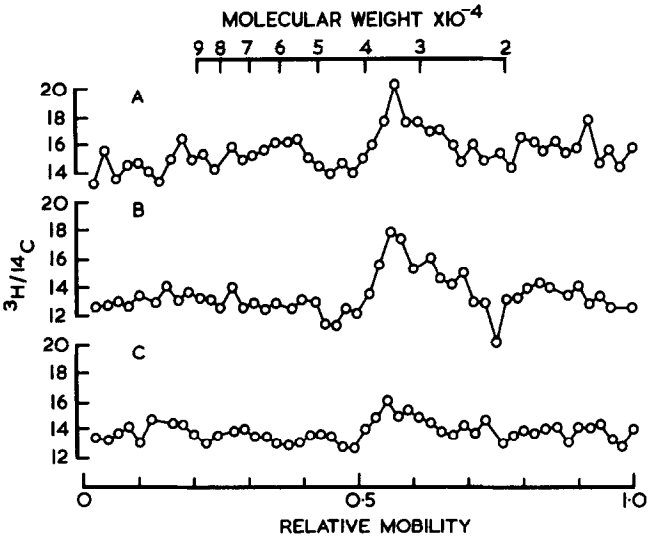

Figure 3. Ratio of $^3H/^{14}C$ radioactivity in nuclear proteins of culture pairs stimulated with A. GARG (^3H) and PBS (^{14}C); B. Con A (^3H) and PBS (^{14}C), and C. Con A (^3H) and GARG (^{14}C).

gels, an experiment was performed in which ^{14}C-pulsed GARG-stimulated and ^3H-pulsed Con A-stimulated cultures were mixed and the ratios of $^3H/^{14}C$ plotted vs relative mobility. The increased ratio seen when GARG/PBS or Con A/PBS combinations were assayed was greatly reduced in the Con A/GARG mixture (Fig. 3), suggesting that the same protein or proteins with very similar mobilities on SDS-PAGE were being affected by the two ligands.

DISCUSSION

We have shown that within 4 hr of in vitro stimulation with a heterologous anti-Ig serum, rabbit PBL undergo a marked change in the synthesis of nonhistone nuclear proteins, especially in the molecular weight range 30-40,000. There is a fast-growing body of evidence implicating nonhistone nuclear proteins in the regulation of gene expression in eukaryotic cells. Increases in synthesis and phosphorylation of nonhistone nuclear proteins have been reported at early times following mitogen stimulation of lymphocytes from several sources (5, 6, 11). Changes in nonhistone nuclear proteins have also been demonstrated in other cell types following neoplastic transformation (12), hormone contact, and at certain stages in the cell cycle (7). A direct demonstration of the ability of nonhistone nuclear proteins to regulate the

synthesis of histone proteins and messenger RNA in HeLa cells has recently been reported (13).

We chose the model system of rabbit PBL stimulated with anti-Ig because it allows us to investigate the results of an interaction of ligand with the putative antigen receptor on lymphocytes, and at the same time stimulate a large percentage of the cell population involved. It is noteworthy that both GARG stimulation, involving only surface heavy and light chain Ig molecules, and Con A stimulation, presumably involving other surface molecules besides Ig (14), result in the same pattern of early nonhistone nuclear protein synthesis. Along with the evidence discussed above, the similarity in results with the two different stimuli suggest that we are observing a basic step in the chain of events involved in transforming a cell from a resting state to mitosis, regardless of cell type or stimulus. Why crosslinking of surface Ig on rabbit PBL should be sufficient to trigger the cells into mitosis when it is not sufficient in murine lymphocytes remains a puzzle, and it will be interesting to see if the same nuclear events occur in LPS- and Con A-stimulated murine lymphocytes as in anti-Ig stimulated rabbit lymphocytes. It is conceivable that cell activation initiated by combination of ligand with surface Ig requires the presence of a complex composed of recognition, transducer and effector elements (15). The minimal explanation for the activation of rabbit PBL by anti-Ig is that they possess a complete complex. Murine B cells, in contrast, might lack an effective linkage between receptor and effector units.

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