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Recruitment of OKM1 staining lymphocytes with selective binding to K-562 tumour targets by interferon

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SUMMARY

Spontaneous cytotoxicity of human lymphocytes for tumours is increased by interferon (IFN) without change in the overall fraction of cells binding to targets. We developed an indirect immunofluorescent technique to stain lymphocytes conjugated to K-562 tumour cells in agarose with monoclonal antibodies. This allowed assessment of lymphocyte subpopulations binding to tumour cells without disruption of conjugates. Overall binding of non-adherent (NA) lymphocytes to tumour targets following incubation at 37°C for 6 h was $13{\cdot}3 \pm 0{\cdot}3\%$ compared to $12{\cdot}5 \pm 0{\cdot}7\%$ with inclusion of IFN at 100 u/ml. When NA lymphocytes were incubated with K-562 tumour cells without IFN, OKM1 and OKT3 staining lymphocytes comprised $16{\cdot}8 \pm 3{\cdot}5\%$ and $83{\cdot}0 \pm 1{\cdot}3\%$ of the total lymphocyte population and $32{\cdot}5 \pm 1{\cdot}3\%$ and $70{\cdot}2 \pm 2{\cdot}6\%$ of lymphocytes conjugated to tumours. Incubation with IFN significantly increased OKM1 staining cells in the total NA population to $57{\cdot}2 \pm 5{\cdot}6\%$ ($P < 0{\cdot}01$) and within tumour conjugates to $59{\cdot}2 \pm 2{\cdot}7\%$ ($P < 0{\cdot}01$) while OKT3 staining cells decreased to $58{\cdot}3 \pm 5{\cdot}2\%$ ($P < 0{\cdot}02$) and $45{\cdot}3 \pm 1{\cdot}2\%$ ($P < 0{\cdot}001$), respectively. IFN increased cytotoxicity of NA cells for ^{51}Cr-labelled K-562 by 66% at an effector to target ratio of 30:1 ($P < 0{\cdot}001$). These results demonstrate that OKM1 staining cells bind more avidly to tumour targets in the absence of IFN. IFN selectively increases the proportion of OKM1 staining lymphocytes with a concomitant increase in their binding to tumour cells. Therefore, enhancement of cytotoxicity by IFN in the NK system may result, in part, from conversion of OKT3 to OKM1 staining cells which are more efficient killers.

INTRODUCTION

Natural killer (NK) cells, effecting spontaneous lysis of tumour cells and other targets (Herberman, 1980; Pross & Baines, 1977; Jondal, Spina & Targan, 1978; Heberman & Holden, 1978; Reitmuller, Wernet & Cudkowicz, 1978), represent a non-specific line of immune defence (Herberman, 1980; Reitmuller *et al.*, 1978). These cells are non-phagocytic, non-adherent, and possess receptors for the Fc fragment of IgG although they interact with targets independent of antibody (Herberman, 1980; Herberman & Holden, 1978; Reitmuller *et al.*, 1978; Herbeman *et al.*, 1979; Kay *et al.*, 1977). Importantly, NK cells also bind OKM1, a monoclonal antibody reacting with mononuclear phagocytes and a subpopulation of T cells (Zarling & Kung, 1980; Kay & Horwitz, 1980). Functional roles that have been ascribed to the NK system include surveillance and killing of

Abbreviations: Ab = antibody; BSA = bovine serum albumin; FCS = fetal calf serum; HBSS = Hanks' balanced salt solution; IFN = interferon; LGL = large granular lymphocytes; NA = non-adherent; NK = natural killer; PBMC = peripheral blood mononuclear cells; Fcγ = lymphocytes with receptors for the Fc portion of IgG.

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tumour cells and virally infected cells and regulation of proliferation and differentiation of systems requiring rapid cell renewal (Herberman, 1980; Reitmuller *et al.*, 1978; Herberman & Ortaldo, 1981; Santoli, Trinchieri & Lief, 1978).

Currently, control of the NK system is not fully understood. Both exogenous and endogenous agents have been implicated as potential regulators either directly or through the effects of potent mediators (Reitmuller, *et al.*, 1978; Herberman *et al.*, 1979). Interferon (IFN) has become recognized as a key immunoregulatory signal for NK activity (Bloom, 1980). IFN markedly augments cytotoxicity of NK cells for tumour targets (Trinchieri & Santoli, 1978; Zarling *et al.*, 1979; Gidlund *et al.*, 1978; Djeu *et al.*, 1979). The increased tumour lysis promoted by IFN does not occur by changes in the overall binding of lymphocytes to tumors, despite the requirement for IFN during the binding step (Silva, Bonavida & Targan, 1980). IFN, thus may recruit precursors to NK cells (pre-NK cells) to bind to tumour targets which then differentiate into effector cells (Silva *et al.*, 1980; Targan & Dorey, 1980). The nature of the subpopulation of lymphocytes recruited for tumour binding by IFN is unknown and the subject of the current report.

A single cell assay in agarose has permitted microscopic evaluation of the interaction of conjugates of single effector and target cells facilitating assessment of mechanisms in this system (Grimm & Bonavida, 1979). We modified this assay to allow indirect immunofluorescent monoclonal antibody (Ab) staining of lymphocytes within lymphocyte–tumour conjugates without disruption of these conjugates. We then used this methodology to study the effect of IFN on the binding of lymphocyte subpopulations to K-562 tumours. The data show that OKM1 staining cells bind more avidly to tumour targets in the absence of IFN. With IFN stimulation, the proportion of OKM1 staining cells increases in the total lymphocyte population and within tumour conjugates.

MATERIALS AND METHODS

Subjects. All subjects were healthy hospital or University employees between the ages of 20 and 40.

Target cells. K-562, a human myeloid line (kindly provided by Dr J. Minowde of Rosewell Park Memorial Institute) was maintained in RPMI 1640 with 10% heat-inactivated fetal calf serum (FCS). This tumour is a sensitive target for natural killer cells (Jondal *et al.*, 1978; West *et al.*, 1977).

Effector cells. Peripheral blood mononuclear cells (PBMC) were prepared by Ficoll-Hypaque sedimentation of heparinized blood (Böyum, 1968). They were depleted of adherent cells by sequential incubation in plastic Petri dishes (100×20 mm, Falcon Laboratories, Oxnard, California, USA) for 1 h at 37°C in 5% CO_2 and on 600 mg acid washed nylon wool columns for 30 min (Greaves & Brown, 1974). The resulting non-adherent T lymphocyte enriched (NA) population consisted of $>85\%$ neuraminadase sheep rosetting cells, $<1\%$ surface immunoglobulin bearing cells, and $<0{\cdot}5\%$ monocytes (cells with non-specific cytoplasmic esterase activity).

Effector–target cell conjugation. This method was a modification of that described by Grimm & Bonavida (1979). Equal numbers of effectors and targets (0·5 ml each of a 2×10^6 cells/ml concentration in RPMI 1640 plus 10% FCS) were incubated in 12×75 mm round bottom plastic test tubes (Falcon Laboratories), at 37°C in 5% CO_2 for 6 h. IFN (lymphoblastoid interferon, HUIFN-α, Batch LNS 77/3A, Burroughs Wellcome, Beckenham, England) at 100 u/ml was added in appropriate experiments at the outset of incubation. These conditions and time of incubation for IFN had been shown to be optimal in promoting cytotoxicity (Zarling *et al.*, 1979; Djeu *et al.*, 1979; Silva *et al.*, 1980). After incubation, the cell mixtures were centrifuged at 250 ***g*** for 5 min at room temperature to allow conjugation. The supernatant was aspirated and 0·5 ml of RPMI 1640 was added as medium without disruption of the pellet. The pellet then was gently resuspended 10 times by pipetting with a 9 inch × 7·0 mm OD Pastuer pipette (VWR Scientific Inc., San Francisco, California). One drop of the cell suspension was placed on a microscope slide with a cover slip and the % conjugates (conjugated lymphocytes) was scored by determining the number of single lymphocytes bound to tumour targets per 300 total lymphocytes counted. The remainder of the cell suspension was retained for monoclonal antibody (Ab) staining.

Monoclonal Ab staining of lymphocytes. Agarose (0·5%) (Accurate Chemical Corp., Hicksville, New York, USA) in Hank's balanced salt solution (HBSS) (KC Biological Assoc., Lenexa, Kansas, USA) was melted at 70°C and then maintained liquified at 40–42°C in water bath. Cell suspension (0·5 ml) was added to 1 ml of agarose, gently mixed, and then 50 μl was pipetted into test tubes (6 × 50 mm, Kimble, Div. of Owens-Illinois, Toledo, Ohio, USA) in triplicate and allowed to cool to room temperature. OKM1, OKT3, OKT4, OKT8 monoclonal Ab (Ortho Diagnostic System Inc., Raritan, New Jersey, USA) were added to appropriate tubes as 50 μl of a 1:20 dilution in HBSS plus 3% bovine serum albumin (BSA) (Sigma Chemical Co., St Louis, Missouri, USA). The tubes were maintained at 4°C for 30 min. Thereafter, 0·5 ml of HBSS plus 5% BSA was added for 5 min and then carefully aspirated from the tubes without disrupting the agarose. In the dark, 50 μl of fluorescent $F(ab')_2$ goat, anti-mouse IgG fragment at a 1:10 dilution was added for 30 min at 4°C. The tubes were again washed with HBSS plus 5% BSA as described, 0·5 ml HBSS and 0·5 ml of 2% paraformaldehyde (Fisher Scientific Co., Fairlawn, New Jersey) were added for 15 min at 4°C for fixation. The tubes were then rewashed with 0·5 ml HBSS for 5 min and the supernatants aspirated. To prepare for fluorescence microscopy, the test tubes were placed in a 70°C water bath to remelt the agarose. At this point, the % conjugated lymphocytes were recounted as detailed above and determined to be equivalent to the pre-monoclonal Ab staining process thus demonstrating integrity of the conjugates through the staining and remelting method. One drop of this suspension was placed on a microscope slide and quickly covered with a cover slip and at ×400 the % fluorescent cells were determined both in the total NA populations (i.e., both single lymphocytes and those within conjugates) and within conjugates. Two hundred lymphocytes were counted in each case.

^{51}Cr release assay. K-562 was ^{51}Cr-labelled by incubating $2·3 \times 10^6$ cells in 0·15 mCi sodium ^{51}chromate (specific activity 1 mCi/ml, New England Nuclear, Boston, Massachusetts, USA) in normal saline for 90 min at 37°C in 5% CO_2. The labelled cells were then washed three times and resuspended in RPMI 1640 plus 10% FCS at a concentration of 10^5 cells/ml. Effector cells were loaded in triplicate into round bottomed wells of microtitre trays with 5×10^3 Cr-labelled K-562 targets at effector to target ratios of 7·5:1, 15:1 and 30:1 in a total volume of 150 μl. The trays were centrifuged at 50*g* for 5 min and incubated for 4 h at 37°C in 5% CO_2. They then were centrifuged at 150*g* for 10 min after which a 50 μl aliquot of the supernatant was harvested into glass test tubes for counting in a gamma counter. Percentage cytotoxicity was quantified by the formula:

$$\frac{\text{ct/min (experimental release)} - \text{ct/min (spontaneous release)}}{\text{ct/min (total release)} - \text{ct/min (spontaneous release)}} \times 100.$$

Spontaneous ^{51}Cr release from target cells was determined in medium alone, while the total release was determined by lysis with detergent (1% sodium dodecyl sulphate). In all experiments, the spontaneous release was less than 10·0% of the total releasable ^{51}Cr. IFN was added in appropriate experiments.

Statistics. The Student's *t*-test was used to determine the significance of differences.

RESULTS

Overall binding of lymphocytes to K-562

In three experiments, 13·3% of non-adherent lymphocytes bound to K-562 tumour cells. Pre-incubation with IFN at a concentration of 100 u/ml for 6 h did not change the overall binding as the % conjugated lymphocytes was 12·5%. The lack of increase in conjugate formation corroborated earlier observations (Silva *et al.*, 1980; Targan & Dorey, 1980).

Monoclonal Ab staining of single and conjugated lymphocytes

Study of the binding of lymphocyte subpopulations to tumours required development of a technique for staining conjugates in agarose in order to prevent their disruption during the washing step (see Materials and Methods). We assessed the effect of IFN on the binding of lymphocyte subpopulations to K-562 using this method for indirect immunofluorescent monoclonal Ab

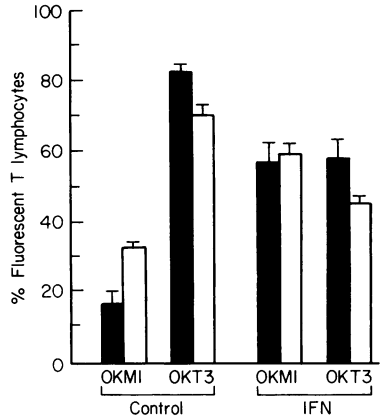

Fig. 1. Monoclonal antibody staining of lymphocytes and conjugates in agarose. Control lymphocytes and IFN stimulated lymphocytes were incubated for 6 h with K-562 tumours. Conjugates were formed and placed into agarose for the purpose of indirect immunofluorescent staining. Lymphocytes within the total NA lymphocyte population and within conjugates are expressed as mean ± s.e. (mean) for three experiments. ■ = total T cell population; □ = T cells with conjugates.

staining of lymphocytes in agarose. OKM1 and OKT3 staining lymphocytes represented 16·8% and 83·0% of the total NA lymphocyte population (Fig. 1). In comparison, OKM1 and OKT3 staining cells within conjugates accounted for 32·5% ($P < 0·02$) and 70·2% ($P < 0·02$) respectively. Thus, in the absence of IFN, OKM1 staining cells bind more avidly than OKM1 non-staining cells to K-562 tumours. Incubation with IFN at 100 u/ml for 6 h significantly increased the number of OKM1 staining cells in the total NA population to 57·2% ($P < 0·01$) and within conjugates to 59·2% ($P < 0·01$). The nearly four-fold increase in OKM1 staining cells in the total NA lymphocyte population following stimulation by IFN, contrasted with a two-fold increase in their binding to tumour target cells. Following IFN exposure, $OKM1^+$ cells did not show selective binding to targets as compared to $OKM1^-$ cells. OKT3 staining cells significantly decreased to 58·3 and 45·3% in the total NA cells and within conjugates respectively following IFN stimulation. After IFN stimulation, the number of OKM1 and OKT3 staining lymphocytes in the total NA lymphocyte population accounted for slightly greater than 100% of the fluorescent cells. IF did not significantly

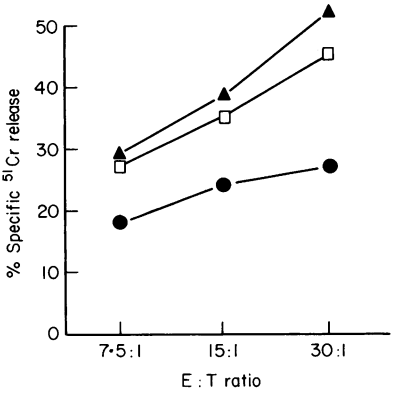

Fig. 2. Effect of interferon on the spontaneous cytotoxicity of NA cell for K-562 using a ^{51}Cr release microcytotoxicity assay. K-562 tumours were labelled with ^{51}chromate and mixed with lymphocytes to establish indicated E:T ratios. After 4 h, supernatants were harvested, counted in a gamma counter and the % specific ^{51}Cr release calculated. The data are expressed as the mean of three experiments. ● = control; □ = IFN (100 u/ml); ▲ = IFN (1,000 u/ml).

change the percentage of OKT4 or OKT8 cells in the total NA lymphocyte populations or in conjugates (data not shown).

Cytotoxicity in a ^{51}Cr release assay

Spontaneous cytotoxicity of NA lymphocytes for K-562 tumours was augmented by IFN in a graded fashion at increasing E:T ratios (Fig. 2). The concentration utilized in the single cell assay, IF 100 u/ml, significantly increased the % specific ^{51}Cr released at each effector to target ratios ($P < 0{\cdot}01$). Percentage specific ^{51}Cr release increased by 66% in the presence of IFN 100 u/ml at an effector–target ratio of 30:1 ($P < 0{\cdot}001$). No significant difference was noted in the augmentation of cytotoxicity by IFN 1,000 u/ml in comparison to IFN 100 u/ml. Thus, the IFN concentration used in the studies of conjugate formation (100 u/ml) produced near maximal effects on cytotoxicity.

DISCUSSION

Monoclonal antibodies reacting with cell surface antigenic determinants can be used to characterize subpopulation of lymphocytes (Reinherz *et al.*, 1979; Kung *et al.*, 1979), and have provided valuable insight into their cellular derivation and functional capacities (Zarling & Kung, 1980; Kay & Horwitz, 1980; Reinherz *et al.*, 1979; Kung *et al.*, 1979; Reinherz & Schlossman, 1980; Reinherz *et al.*, 1980). NK cells react with OKM1 antibody which also identifies a surface antigen found on monocytes and granulocytes (Zarling & Kung, 1980; Kay & Horwitz, 1980; Timonen, Ortaldo & Herberman, 1981; Breard *et al.*, 1980). Van de Griend *et al.* (1982) recently demonstrated that OKM1 staining cells, which were non-reactive with OKT3 antibody, accounted for the majority of IgG, Fc receptor bearing lymphocytes (Fcγ) and displayed prominent NK activity. A subpopulation of Fcγ cells were $OKM1^+$ and $OKT3^+$ but displayed poor NK activity. Rubin, Pross & Roder (1982) using a single cell assay of effector cell conjugation to tumours recently reported that OKM1, Mac-1 and HNK-1 reactive cells showed increased binding to K-562 in comparison to cells lacking these markers.

IFN enhances NK activity without a change in the total binding of effector to target cells in part by recruiting a precursor to the NK cell to bind to tumours and differentiate into effector cells (Silva *et al.*, 1980; Targan & Dorey, 1980). IFN did not effect the binding of large granular lymphocytes (LGL), a subpopulation with major NK activity (Timonen *et al.*, 1981) to K-562 (Timonen *et al.*, 1982); however, IFN increased the rate of tumour lysis and also activated killing by some non-lytic binding LGL (Timonen *et al.*, 1982). Zarling & Kung (1980) demonstrated that depletion of OKM1 reactive cells significantly diminished NK activity in cells stimulated by polyinosinic:polycytidylic acid, a potent inducer of IFN. However, the nature of the cells recruited by IFN to bind to tumour targets had not been established directly.

In the present studies, an indirect immunofluorescent technique was developed to stain lymphocytes conjugated to K-562 tumours with monoclonal antibodies; this technique allowed evaluation of lymphocyte subpopulations binding to tumour targets. This procedure offered major advantage over methodology reported previously: staining and washing conjugates in agarose prevented their disruption. We found that in the absence of IFN, OKM1 staining lymphocytes showed greater avidity for tumour targets than did cells lacking this marker. Stimulation with IFN significantly increased OKM1 staining cells within the NA cell population and concomitantly within conjugates. Since OKM1 and OKT3 staining cells accounted for more than 100% of NA cells following IFN stimulation, dual markers may exist on a minor subpopulation of lymphocytes. We conclude that IFN recruits $OKT3^+$, $OKM1^-$ cells to express the OKM1 marker primarily. The functional consequence of this cellular differentiation is enhanced natural killer cell activity.

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