Preservation of in Vitro Biological Functions in Regional Lymph Node Lymphocytes in Squamous Head and Neck Cancer

Andrew Saxon and Joe Portaro

Department of Microbiology and Immunology, Immunobiology Group, UCLA School of Medicine, Los Angeles, California 90024

SUMMARY

Regional lymph node lymphocytes from patients with squamous cancer of the head and neck were tested in vitro for their ability to proliferate in response to phytohemagglutinin, concanavalin A, and allogeneic stimuli in one-way mixed lymphocyte culture. Their ability to act as cytotoxic effectors in phytohemagglutinin-dependent cellular cytotoxicity was also evaluated, and all results were compared to normal lymph node or blood lymphocytes. The regional lymph node lymphocytes retained proliferative capabilities equal to those in control lymph nodes or blood, whereas they were unable to mediate phytohemagglutinin-dependent cellular cytotoxicity. However, this was not a tumor-related effect because normal lymph node lymphocytes were also ineffective in this assay. The failure of the regional immune response to control early tumor growth could not be accounted for by generalized nonspecific immunosuppression in regional lymph node lymphocytes, inasmuch as these cells demonstrated normal in vitro activity.

INTRODUCTION

The regional immune response, as represented by draining RLN, will be the 1st level of interaction between a developing neoplasm and the host's immune capabilities. This occurs because tumor cells and/or tumor-related products will be drained into regional lymphatics prior to vascularization of the tumor. The continued growth of the neoplasm might be related to an absolute lack of stimulation of the host's immune response (nonimmunogenicity). However, previous work showing changes in lymphoid subpopulations as well as evidence of histological alterations (13) as well as evidence of generalized immunosuppression in RLN will be exposed to high concentrations of tumor products from a very early stage in tumor development. Blood lymphocytes from patients with cancers have been reported to demonstrate such nonspecific immunosuppression both in vivo and in vitro (6, 10).

To test the hypothesis that the regional immune response may show generalized immunosuppression of reactivity, RLN lymphocytes from patients undergoing primary tumor surgery with RLN dissection were tested in PHA, Con A, and 1-way MLC-proliferative assays. The responsiveness of these RLN lymphocytes was compared to that for autologous DLN, NLN, and PBL. These same lymphocyte populations were also tested for their ability to function in short-term (2- to 3-hr) PDCC. These studies demonstrated no evidence of generalized immunosuppression in RLN, because RLN lymphocytes retained in vitro capabilities similar to those of their normal counterparts.

MATERIALS AND METHODS

Patients, Preparation and Separation of Lymphoid Suspensions, and Analysis of Surface Receptors. These procedures and results are described in detail in the preceding paper (13).

Mitogen Stimulation. For mitogen stimulation studies, lymphocytes were suspended at a concentration of 1 × 10⁶/ml in RPMI 1640 media supplemented with streptomycin, penicillin, 10% human AB serum (heat inactivated), and L-glutamine. One-tenth ml of this suspension (1 × 10⁵ lymphocytes) was added to the wells of Cooke microtiter plates (Grand Island Biological Co., Grand Island, N. Y.), and 0.1 ml of supplemented RPMI 1640 media with or without PHA or Con A was added. All tests were run in triplicate. The plates were incubated with 5% CO₂ in a 37° humidified incubator. Twenty-four hr prior to harvesting, each well was pulsed with 2 μCi of [³H]thymidine (New England Nuclear, Boston, Mass.) in 20 μl of RPMI 1640 media. The plates were harvested, and incorporated [³H]thymidine was counted using a liquid scintillation system.

Lectin-dependent Cell-mediated Cytotoxicity. Several murine tumor lines were used as target cells to determine the lectin-dependent cellular cytotoxicity of lymphoid cells: D6 (Gross virus-induced lymphoma) syngeneic in C3H mice; EL-4 leukemia syngeneic in C57BL/6 mice; and G-35 (Gross virus-induced lymphoma) syngeneic in BALB mice. D6 was grown in suspension in Tissue Culture Medium MJLA (Microbiological Associates, Bethesda, Md.) supplemented with 10% heat-inactivated fetal calf serum. G-35 and EL-4 were maintained in ascites form. Immediately before test-
ing, the target was labeled with $^{31}\text{Cr}$ using the technique of Wigzell (18). Sodium chromate, 0.4 µCi, was added to $10^7$ tumor cells in 1 ml of media supplemented with 10% fetal calf serum. After incubation at 37° for 90 min, the targets were washed 4 times with media and added to the effector lymphocytes with an effector:target ratio of 20:1 in a final volume of 0.2 ml. For D6, $1 \times 10^4$ targets were used per tube; for EL-4 and G-35, $2.5 \times 10^4$ targets were used per tube. PHA was suspended in media to the appropriate concentration ($\mu$l/ml) and added in 0.1 ml. The tubes were centrifuged at 200 x g for 5 min and incubated for 2 hr at 37° in a 5% CO$_2$-humidified atmosphere. The reaction was stopped by addition of 1 ml of cold media to each tube. After centrifugation at 800 x g for 10 min, the supernatants were removed, and radioactivity in the supernatants was determined in a gamma counter. Specific lysis was calculated according to the formula:

$$\% \text{specific lysis} = \frac{\% \text{Cr released in experiment} - \% \text{Cr in minimum essential medium}}{\% \text{Cr released by detergent} - \% \text{Cr released in minimum essential medium}}$$

The percentage of radioactivity released by detergent represents the total lysable count (80% average). All tests were set up in triplicate.

MLC. One-way MLC was performed in triplicate in Cooke microtiter plates. Two $\times 10^6$ responder lymphocytes were added to each well in 0.1 ml of RPMI 1640 media supplemented with L-glutamine, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer, gentamicin, and 20% human AB serum (complete RPMI). Stimulator cells were treated with mitomycin C (Sigma Chemical Co., St. Louis, Mo.). Lymphocytes ($15 \times 10^6$) were suspended in 1.0 ml of RPMI 1640 containing 50 µg of mitomycin C, incubated for 37° with stirring for 30 min, and washed 3 times with 0.15N phosphate-buffered saline. After resuspension in complete RPMI, $2 \times 10^6$ lymphocytes were added to the test wells in triplicate in 0.1 ml of media. The MLC plates were pulsed with $[^3\text{H}]$thymidine and harvested in a manner identical with the mitogen stimulation plates. Controls consisted of responder cells alone, mitomycin-treated stimulator cells alone, and allogeneic mitomycin-treated cells added together.

Statistical Analysis. Statistical analysis of PHA and Con A proliferation was performed using Student's t test (2-way).

RESULTS

Proliferative Response of RLN Lymphocytes following Stimulation with PHA. The ability of RLN and NLN lymphocytes to proliferate in response to PHA was examined using final PHA dilutions of 1:10, 1:100, 1:1000, and 1:2500. Samples were examined on Days 2 through 6 (Chart 1). The maximal response was seen at a 1:100 dilution for each of 2 NLN and 2 RLN. A total of 3 NLN and 3 RLN were tested for their kinetic response to PHA, (1:100) and all demonstrated a maximum response between Days 3 and 4 with a gradual decline in $[^3\text{H}]$thymidine incorporation thereafter (Chart 2).

To test the relative reactivity of LNL and PBL to PHA, a total of 8 NLN and 12 RLN were tested on Day 3 for proliferation after stimulation with PHA (1:100) (Chart 3). Each sample was tested simultaneously with a sample of normal PBL from an age-matched control. Both groups demonstrated a level of proliferation similar to that for normal PBL (although RLN and NLN lymphocytes were not tested simultaneously). No RLN or NLN demonstrated a markedly depressed response to PHA. However, RLN from the 2 patients with squamous cancer of the head and neck who received preoperative radiation showed a decreased PHA response (Chart 3). The average background reactivities for RLN, NLN, and PBL as assayed on Day 3 without the addition of PHA were 1463, 1239, and 1051 cpm, respectively.

Con A Response. The ability of NLN and RLN lymphocytes to incorporate $[^3\text{H}]$thymidine in response to Con A was evaluated on Day 3 using $10 \mu$g of Con A per ml, conditions previously found to be optimal for stimulation of PBL (12).
the same patients were capable of lysing the targets in the presence of PHA (Chart 5). These same LN cells were shown to be able to proliferate normally at 3 days in response to PHA stimulation. T- and B-cell fractions from several of the same LN preparations were also tested for their ability to mediate PDCC, and both fractions remained relatively inactive (Chart 5). The ability to mediate PDCC can be found in both the T- and B-cell fractions from normal PBL separated in this manner (B. Bonavida, personal observation). For removal of any lymphocyte membrane-bound material that might block the PDCC activity of these LN L, LNL were washed 12 times in media after incubation at 37° for up to 1 hr. This procedure did not increase PDCC by LN cells, nor did incubation of the LN lymphocytes at 37° for up to 24 hr prior to testing.

LNL Dose Response and Kinetics in 1-Way MLC. One-way MLC was established using stimulator:responder cell ratios of 1:2, 1:1, and 2:1. NLN and RLN lymphocytes responded equally well at the 1:2 and 1:1 ratios with only a minor decrease in [3H]thymidine incorporation at the 2:1 ratio. Normal PBL showed a clearly superior response at the 1:1 ratio; this ratio was therefore used for subsequent studies. The proliferative response by LNL was relatively constant on Days 3 through 6 with some decline thereafter (data not shown). For further studies, cultures were tested on Day 5 inasmuch as this has been shown to be the time of maximal PBL response in the MLC (19).

Comparison of Reactivity of NLN and RLN Lymphocytes to Allogeneic Stimuli in MLC. NLN and RLN lymphocytes were tested fresh against 2 allogeneic sets of mitomycin C-treated PBL. The response of a nonrelated normal PBL against each stimulator cell was taken as baseline, and the ratio between the LNL response and PBL response to the allogeneic stimulus was calculated.

\[
\text{Response index} = \frac{\text{cpm (LNL)}}{\text{cpm (PBL)}}
\]

The mean [3H]thymidine uptake for 3 NLN was 135,932 ± 16,855 (S. D.) for 3 RLN was 143,469 ± 15,063, and for 4 normal PBL it was 140,954 ± 20,860. Again no clear difference between the various lymphoid populations was demonstrated with the concentration of Con A used.

LNL Ability to Mediate PDCC. All RLN and NLN lymphocytes demonstrated either an absent or markedly diminished ability to mediate PDCC against the D6 target as compared to PBL from age-matched controls (Chart 4). Therefore, the ability of PBL from the patients was compared to their LNL with regard to their ability to act as effectors in PDCC. For these studies, EL-4 and G-35 targets were used. Again both NLN and RLN cells did not demonstrate the ability to act as effectors in PDCC, while PBL from
The strength of the allogeneic stimulus will vary between pairs of stimulator and responder cells, making misleading individual comparisons between reactivity by NLN lymphocytes from noncancer patients and RLN lymphocytes from cancer patients toward one given allogeneic stimulus. Thus, the general reactivity between the 2 groups was compared (Chart 6). RLN and NLN lymphocytes responded in a fashion similar to that of the allogeneic PBL stimulus. NLN or nonradiated RLN cells all demonstrated at least a 10-fold increase in \[^{[\text{H}]}\text{thymidine incorporation as compared to the same unstimulated lymphocytes. However, RLN obtained from 2 patients who had received local radiation therapy showed a clearly decreased responsiveness (Chart 6).}

**Comparison between RLN and DLN Lymphocyte Reactivity in MLC.** RLN and DLN lymphocytes from the same cancer patient were compared for their ability to respond in 1-way MLC. Since both LN were from the same individual, comparisons are valid between RLN and DLN response to a given allogeneic stimulus. In 4 of 6 pairs tested, the RLN lymphocytes showed a decreased proliferation in the MLC when compared to DLN lymphocytes (Table 1). This differential reactivity was always present for the pair of allogeneic stimuli. In 1 pair of RLN and DLN tested no difference in reactivity was found, while in the other pair RLN cells reacted somewhat better than did DLN cells. Again no LNL from any untreated patient were found that did not respond well to allogeneic stimuli. We did examine RLN and DLN from 2 patients with squamous cancer of the head and neck who had undergone radiation therapy. In each case the radiated RLN and DLN lymphocytes demonstrated a marked depression in alloreactivity (Table 1).

**DISCUSSION**

The cells that proliferate in response to stimulation with PHA and Con A have been demonstrated to be overlapping subpopulations of T-cells (15). Animal LN cells and PBL have been shown to respond to these mitogens equally well (16). Our data demonstrate that human NLN lymphocytes also show a degree of lectin-induced proliferation comparable to that of PBL. RLN lymphocytes from patients with early melanoma or squamous head and neck cancer did not differ in maximal \[^{[\text{H}]}\text{thymidine incorporation from either NLN lymphocytes or normal PBL. RLN that had been radiated, however, showed a diminished PHA response. The equality in RLN and NLN response was in spite of the fact that RLN had an average of 21% less T-cells by sheep ABC rosette testing. Indeed, if the cpm \[^{[\text{H}]}\text{thymidine per number of T-cells are calculated for our data, then RLN lymphocytes were more reactive on this basis than either normal LN cells or PBL. The suggestion that RLN T-cells may be activated in vivo and therefore show increased reactivity is supported by the observation that RLN had elevated levels of lymphocytes that form spontaneous rosettes with human RBC (A. Saxon, personal observation). This surface marker has been reported to characterize activated T-cells (14). However, our data can be manipulated to show a decreased response to mitogens in RLN by calculating a stimulation index (cpm with PHA/cpm without PHA). Since the background \[^{[\text{H}]}\text{thymidine incorporation (cpm without PHA) in RLN lymphocytes was 1.4 times greater than in normal PBL and 1.2 times greater than in NLN lymphocytes,}
the stimulation indices would reflect these differences. The significance of the increased background in RLN is not clear. It may represent increased spontaneous DNA synthesis, which has been shown in RLN in animal models (7), or increased trapping of [3H]thymidine within the cytoplasmic pool of RLN lymphocytes. Because of these various possibilities, we have presented our data as maximum cpm and given separately the percentage of cells in the various populations and the background cpm.

The lymphocytes that respond to allogeneic stimulation in MLC are a small population of approximately 2 to 6% of the T-lymphocytes (8). Both NLN lymphocytes from normals and RLN lymphocytes from cancer patients reacted to allogeneic stimuli equally well. Two-thirds of DLN demonstrated an increased reactivity in MLC when compared to RLN from the same individual. Since DLN contained an average of 10% more T-cells, this increased reactivity may simply relate to the presence of an increased number of reactive cells. While NLN contained 20% more T-cells than RLN, differential reactivity may not have been demonstrable because direct comparisons could not be made. Certainly all NLN, RLN, and DLN showed the ability to respond to allogeneic stimuli. RLN and DLN from patients who had received local radiation therapy exhibited MLC reactivity far below that seen with any other lymphoid suspension tested. This is in spite of the fact that these radiated LN had T-cell percentages similar to that seen in nonradiated DLN. Either the subpopulation of T-cells responsible for alloreactivity was specifically diminished by the radiation or, what is more likely, the reactivity of lymphocytes in general was compromised by radiation damage. The latter interpretation is supported by the fact that the PHA response by these radiated RLN was also markedly depressed. An alternative explanation that cannot be excluded is that those patients who received radiation therapy had more extensive tumors and this may have played an undefined role in the observed results.

Cell-mediated lysis of target cells in the presence of PHA (PDCC) occurs in 2 hr and is not dependent on proliferation (3). The lymphocyte subpopulation(s) that mediate this activity remain ill-defined. In normal PBL, both the T-enriched and B-enriched fractions derived by our separation technique can effect PDCC, although the B-enriched fraction is more active. PBL from normals and the cancer patients were effective in the PDCC assay, while LN cells from the same normals and cancer patients were ineffective. These same LN lymphocytes were simultaneously shown to proliferate normally over 3 days in response to PHA. Furthermore, neither the purified T- nor B-populations of LN, RLN, or NLN were capable of lysing targets in the presence of PHA. These data suggest that the cell population(s) responsible for PDCC are either absent or not functional in NLN and RLN. The proliferative response to PHA, Con A, and allogeneic stimuli is a property of T-cell subpopulations (9). The reactivity of NLN in all 3 assays supports the concept that LN T-cells share proliferative capabilities similar to their PBL counterparts. While RLN demonstrated a decreased proportion of T-cells, these RLN retained intact T-cell responder mechanisms as measured by proliferative response to the various stimuli. There may be some decrease in reactivity in MLC related to diminished T-cell numbers, but certainly no generalized immunosuppression of T-cell reactivity was demonstrated in RLN. This agrees with the reports of Burk et al. (4), who reported that LN cells from animals bearing small and large tumors responded to mitogens with intact T-cell proliferation. One must be aware of the fact, however, that microculture systems may give different results than do macroculture systems (10). Lymphocytes from radiated RLN showed decreased PHA and MLC reactivity far out of proportion to any change in the percentage of T-cells present. This correlates with evidence that lymphocytes show diminished reactivity after in vivo and in vitro radiation exposure (5, 17).

Both NLN and RLN lymphocytes were unable to mediate lysis of target cells in the presence of PHA, while PBL from the same donors retained this activity. The lymphocytes responsible for PDCC appear to be compartmentalized outside both NLN and RLN, being found in blood and spleen. A parallel situation occurs in that the cells responsible for antibody-dependent cell-mediated cytotoxicity are not found in NLN or RLN, while they are found in peripheral blood or spleen (11, 12).

Overall, the methods used in this study indicate that RLN from patients with early localized tumors do not demonstrate depressed immune reactive capabilities when compared to NLN. We would propose that inability of RLN to control early growth and local spread of a tumor does not relate to generalized suppression of RLN reactivity but may relate to tumor-specific suppression of LN activity. The tumor may effect this by modulation of the immune response to its own advantage through the generation of host suppressor cells or other blocking agencies that prevent the development or expression of an effective specific immune response.

ACKNOWLEDGMENTS

The authors would like to express their thanks to Dr. John L. Fahey for his encouragement and helpful suggestions in preparing this manuscript, to Dr. Ben Bonavida for performing some of the cytotoxicity assays and for reviewing this manuscript, and to Joanne Bihr for her excellent technical assistance.

REFERENCES

A. Saxon and J. Portaro


Preservation of *in Vitro* Biological Functions in Regional Lymph Node Lymphocytes in Squamous Head and Neck Cancer

Andrew Saxon and Joe Portaro