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Interleukin-10 as a Possible Mediator of Immunosuppressive Effect in Patients with Squamous Cell Carcinoma of the Head and Neck

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Background: Depressed cell-mediated immunity is a frequent event in patients with head and neck cancer and is characterized by impairment of T cell–proliferative responses and natural killer cell and lymphokine-activated killer cell activity. This immunosuppressive effect appears to be mediated by a serum-derived factor. Certain cytokines, including transforming growth factor-beta (TGF-β) and interleukin (IL)-10 have been shown to induce similar immunosuppressive effects. The present study was designed to examine the putative role of these cytokines in cellular immune suppression induced by patient serum.

Methods: Serum was collected from multiple patients with newly diagnosed or recurrent squamous cell carcinoma of the head and neck. The serum was heat inactivated for 30 min and frozen in aliquots. Peripheral blood lymphocytes were isolated from normal human blood. Lymphocytes were suspended in RPMI and 15% concentrations of control and patient serum and stimulated with 0.75 mg% phytohemagglutinin. In addition, neutralizing antibodies to TGF-β and IL-10 were added to lymphocyte cultures. At 24 h, an IL-2 response assay was performed. Finally, the sera were examined for the presence of TGF-β and IL-10 using an enzyme-linked immunosorbent assay (ELISA).

Results: In seven of seven experiments, incubating cells with a neutralizing antibody to TGF-β failed to counteract the immune suppression and restore proliferative response to IL-2. Also, an ELISA of these sera failed to demonstrate the presence of TGF-β. In contrast, four of five experiments performed with neutralizing antibody to IL-10 showed significant restoration of proliferation in the presence of this antibody. Also, ELISA showed elevated IL-10 levels in 65% of the patients' sera in comparison to controls.

Conclusion: We conclude that TGF-β is not responsible for the immunosuppressive effects induced by head and neck patient sera. However, the suppressive effect is reversed by blocking the biologic action of IL-10. Further experiments are needed to define the role of IL-10 in inducing the immunosuppressive effect.

Key Words: Interleukin-10—Immunosuppression—Squamous carcinoma of head and neck.

Depressed cell-mediated immunity is a frequent finding in patients with head and neck cancer. We and others have shown depressions in T cell–proliferative responses, as well as impairment of natural killer (NK) cell and lymphokine activated killer (LAK) cell activity in these patients (1–7). Furthermore, this effect appears to be mediated by a serum-derived factor (7–9) and can be induced by supernatants derived from squamous carcinoma cell lines (10). Recently we showed that there is a correlation between suppression and decreased expression of the IL-2 receptor (11). Numerous cytokines and soluble factors have been suggested as possible mediators of this immunosuppressive effect, including transforming growth factor-beta

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(TGF-β), interleukin (IL)-10, soluble IL-2 receptor, and various prostaglandins (12).

TGF-β has many well-known immunosuppressive effects, including inhibition of NK cell activity, LAK cell generation, and lymphocyte proliferation (13). In addition, it has been isolated from various tumors, including sarcomas and glioblastomas (13), as well as from ovarian ascitic fluid (14) and squamous cell carcinoma of the esophagus (15). Finally, this cytokine has been reported to alter the expression of the P55 IL-2 receptor on activated lymphocytes (16).

IL-10 was first described as cytokine synthesis inhibitory factor after being isolated from murine TH2, or T-helper cells (17). Investigators have characterized this cytokine as a downregulator of cell-mediated immunity, which can suppress the production of numerous proinflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), IL-1, IL-6, and IL-8 (18,19). This cytokine has been isolated from basal cell and squamous cell carcinomas and has been suggested as a possible mediator of local immunosuppression (20). The TH1/TH2 dichotomy has been well described in mice (17,21,22). These two types of T-helper clones also have been described in humans (23), although the dichotomy does not appear to be as clearcut in humans as in mice (24). When activated by antigen/antigen-presenting cells (APCs), Th1 cells produce IL-2, interferon-gamma (IFN-γ), and lymphotoxin (LT), which are critical for mediating cell-mediated responses. In contrast, the TH2 cytokine pattern includes IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13, all of which stimulate B cells and the humoral response (17,21). IL-10 plays a crucial role in suppressing TH1 cell responses and in mediating humoral immunity.

Human IL-10 is an 18-kDa, nonglycosylated, acid-labile protein (17), the gene of which has been isolated from chromosome 1 (25). Murine IL-10 migrates during sodium dodecyl sulfate (SDS) electrophoresis in two major bands corresponding to molecular weights of 17 and 21 kDa (22). It also differs from human IL-10 in that it is highly glycosylated (22). The nucleotide sequence of the open reading frame of human IL-10 is 81% homologous to mouse IL-10, and the mouse sequence is 91% homologous to rat IL-10 (26). Interestingly, the open reading frame for IL-10 has been found to be extensively similar to that of Epstein-Barr virus BCRF1 protein (71% DNA homology and 84% protein homology), and recombinant BCRF1 protein has been shown to mimic the activity of IL-10 (27–29). A second gene with homology to IL-10 is present in the genome of an equine herpesvirus (30). These data suggest that these herpesviruses may exploit the biological activity of the captured cytokine gene to enhance survival in the host.

In addition to inhibition of TH1 immunity, IL-10 has been shown to have numerous other biological effects. In macrophages, IL-10 has been shown to inhibit cytokine synthesis (IL-1, granulocyte-macrophage colony-stimulating factor, TNF, IL-6, IL-8, IL-10, and IL-12) in response to lipopolysaccharide (19). This inhibition is distinct from mechanisms triggered by other suppressive agents, including IL-4 and TGF-β (31). In addition, IL-10 has been shown to inhibit expression of major histocompatibility complex class II antigens on certain classes of monocytes/macrophages (32). This appears to be the mechanism by which IL-10 produces its effect on TH1 cells because cell-free stimulation methods such as anti-CD3 antibodies induce TH1 synthesis of IFN-γ that is not inhibited by IL-10 (17). Also, when TH1 cells are stimulated by non-macrophage APCs (B cells), they are resistant to the effects of IL-10 (18).

Finally, the soluble IL-2 receptor has been suggested as a possible mediator of suppressed immune responses in patients with solid tumors (33,34). We performed a series of experiments to examine the role played by the above factors in the inhibited IL-2 response mediated by the serum-derived factor in patients with squamous cell carcinoma of the head and neck.

METHODS

Blood samples were drawn into serum separator tubes from patients with newly diagnosed or recurrent squamous cell carcinoma of the head and neck. Blood was allowed to clot and centrifuged at 2,500 RPM for 5 min. Sera were collected, heat-inactivated for 30 min at 56°C, and frozen in aliquots for future use. Control sera were gathered from normal individuals. Peripheral blood lymphocytes (PBLs) were isolated from human blood using a Ficoll-Hypaque gradient. Previous experiments had determined suppressive patient sera, and these sera were used in the following experiments (11). Our experiments were conducted on seven patients. Four of the patients presented with TNM stage IV disease, two with stage III disease, and one with

stage II disease. Six of the seven patients were treated with multimodality therapy, which included radiation, chemotherapy (5-fluorouracil [5-FU], and cisplatinin), and surgery (including a functional neck dissection with wide excision of the primary tumor). One patient with a recurrence in the nasal vestibule received brachytherapy in addition to external-beam radiation. The anatomic distribution of the primary lesions included nasal vestibule, maxilla, two supraglottic larynges, lingual tonsil, floor of mouth, and one base of tongue lesion.

Neutralization of Activity Using anti–TGF-β Antibody

Sera from controls and patients were added to peripheral blood lymphocytes at a final concentration of 15%. Each experiment required a minimum of four separate incubation flasks: two for control sera with and without neutralizing antibody and two for patient sera with and without neutralizing antibody. The amount of TGF-β antibody (Pan-TGF-β, chicken immunoglobulin G, R&D Systems, Minneapolis, MN) added, was based on the effective neutralizing dose 50% (ND_{50}) as recommended by the manufacturer. The ND_{50} is defined as that concentration of antibody required to yield one-half maximal inhibition of the cytokine activity on a responsive cell line when that cytokine is present at a concentration just high enough to elicit a maximum response. All cells were then stimulated with 0.75% phytohemagglutinin (PHA) for 24 h. After this period, cell viability was assured with trypan blue, and PBLs were washed twice in saline and resuspended in RPMI with 10% fetal calf serum (FCS), at 10^6 cells/ml. An IL-2 response assay was then performed by incubating cells in triplicate with increasing concentrations of recombinant IL-2 (rIL-2). Thymidine uptake was evaluated at 72 h.

To further examine the role that TGF-β might play in these patients, numerous samples of serum were tested by enzyme-linked immunosorbent assay (ELISA) for TGF-β (R & D Systems). The ELISA uses a quantitative "sandwich" immunoassay technique. The sera were previously determined to be immunosuppressive by IL-2 assay. Samples were then stored at −70°C. Because TGF-β can sometimes be present in a latent form, undetectable by ELISA, selected samples were treated as recommended by the manufacturer. These samples were treated by adding glacial acetic acid to 1 ml of serum for a final concentration of 1 *M* acetic acid. After 1 h at room temperature, the acidified serum was dialized twice against 100 vol PBS. After dialysis the samples were used straight and immunoassays were performed as described.

IL-2 Response of Activated Lymphocytes in Patient Sera

In the following experiment, PBLs were collected from a buffy bag as described above. Cells were suspended in RPMI with 10% FCS at 10^6 cells/ml and stimulated with 0.75% PHA for 7 days. Viability was then assessed with trypan blue. PBLs were counted, washed twice in saline, and resuspended in RPMI with 15% concentrations of patient sera, all of which were found to be suppressive on previous assays (total of 13 patient sera). Cell concentration was 10^6 cells/ml. An IL-2 response assay was performed, as described previously, using increasing concentrations of rIL-2 (1, 10, and 100 U/ml) and incubating in triplicate.

Neutralization of Activity Using Anti–IL-10 Antibody

This experiment was performed as described for neutralizing antibodies to TGF-β. Briefly, PBLs were collected from a buffy bag and resuspended in RPMI with 15% concentrations of control and patient sera, as well as neutralizing antibody to IL-10 (antihuman IL-10, mouse IgG; R & D Systems). Cells were then stimulated with 0.75% PHA for 24 h. PBLs were then assessed for viability using trypan blue, counted, washed twice with saline, and resuspended at 10^6 cells/ml in RPMI with 15% FCS. An IL-2 response assay was then performed as described.

IL-10 ELISA

Finally, numerous samples from patients were examined for IL-10 levels. Sera were collected and stored as previously described. An ELISA using the quantitative sandwich technique (R & D Systems), was used to detect serum levels of IL-10 among our patients. In addition, sera were collected from three normal controls.

RESULTS

Results of the TGF-β neutralizing assay are shown in Fig. 1. In this experiment, sera from patients HN4 and HN15 were found to be more suppressive than those from HN14 and HN17. Addi-

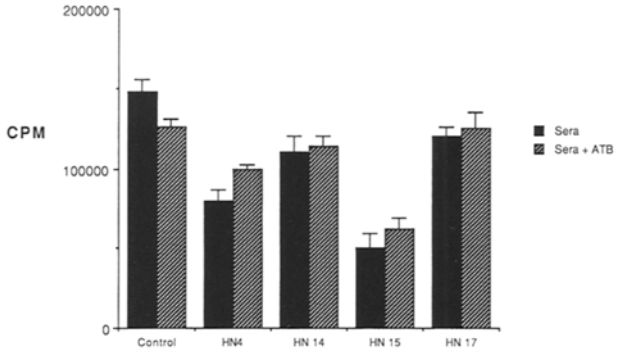


FIG. 1. IL-2 response assay of normal lymphocytes incubated in control and patient serum (HN4–HN17), with and without neutralizing antibody to TGF-β. Error bars represent standard deviations.

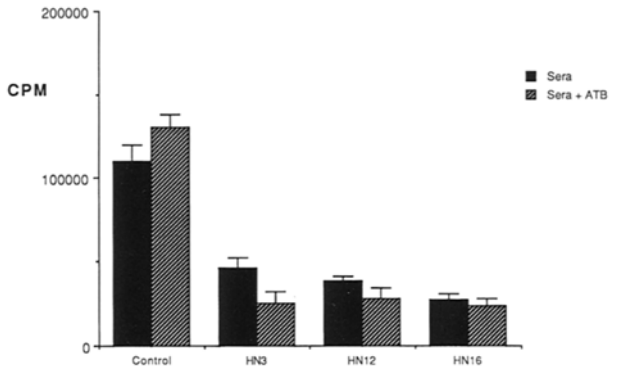


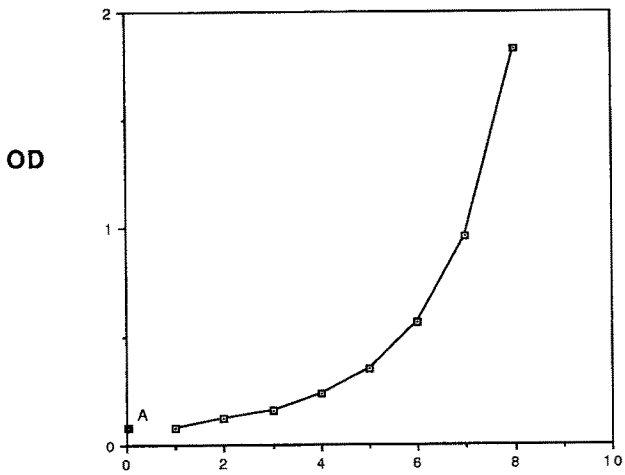
FIG. 2. IL-2 response assay of normal lymphocytes incubated in 15% concentration of control and patient sera (HN3–HN16), with and without neutralizing antibody to TGF-β. Error bars represent standard deviations.

tion of a pan-TGF-β antibody failed to change thymidine uptake among lymphocytes exposed to patient sera. Similar results are shown in Fig. 2, in which significant inhibition was seen in samples from HN3, HN12, and HN16. Of note is the increased inhibition noted in Fig. 2 relative to Fig. 1. Although the control sera are the same in both experiments, the PBLs are different, and this may account for the difference in inhibition. Again, activation of PBLs in the presence of neutralizing antibody to TGF-β failed to reverse the inhibitory effect. In addition, ELISA testing of patient serum was performed for TGF-β. None of the serum samples tested positive for TGF-β. These data are shown in Fig. 3.

Serum effects on fully activated PBLs are shown in Fig. 4. In this experiment, PBLs were activated in FCS, allowed to incubate for 7 days, and resuspended in patient serum at 10^6 cells/ml (see Methods). A total of 13 patient sera were used, and the average values with standard deviations are depicted in our graph. The IL-2 response assay showed no inhibition by the sera once T cells were fully activated. The results of this experiment discount the notion of a soluble IL-2 receptor being a mediator of the immunosuppressive effect.

Neutralizing IL-10 results are shown in Fig. 5. Our graph shows the percentage reconstitution after exposure of cells and sera to neutralizing antibodies to IL-10. Specifically, the second bar in each group depicts the ratio of thymidine uptake of patient sera, with neutralizing antibody as the numerator and control sera with antibody as the denominator. As shown, the inhibitory effects of HN4, HN11, HN12, and HN14 were significantly reversed by the addition of IL-10 antibody. This reversal was not seen in HN15. Overall four of five experiments showed a reversal of the inhibitory effect after treatment with neutralizing IL-10 antibody.

Finally, the results of our IL-10 ELISA are



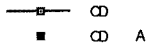


FIG. 3. TGF-β ELISA. Standard curve as shown. Mean value for controls 0.0495 (OD). Mean patient value 0.0475 (OD). Point represents TGF-β levels in control and patient sera. OD, optical density.

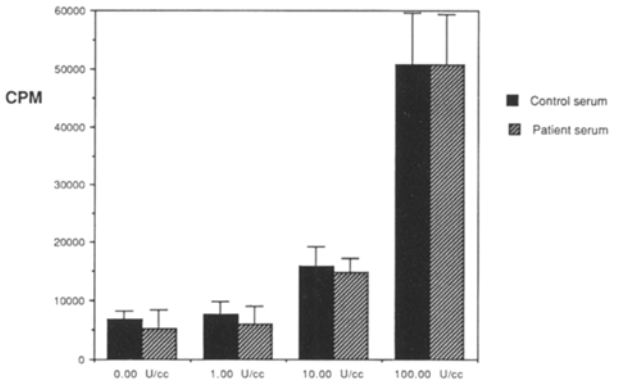


FIG. 4. IL-2 response assay of 7-day PHA-activated T cells in FCS (fetal calf serum), subsequently exposed to head and neck cancer patient serum (HNS, n = 13). Error bars represent standard deviations.

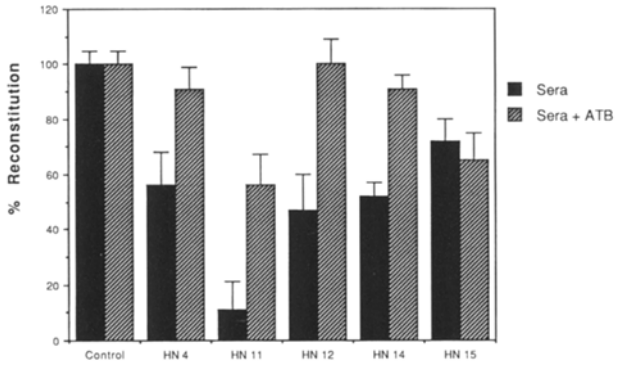


FIG. 5. IL-2 response assay (100 U/ml), of lymphocytes activated in control and patient sera, with and without neutralizing antibody to IL-10. Error bars represent standard deviations.

shown in Fig. 6. Our control value was obtained by averaging the IL-10 levels from three separate control sera. Overall, seven of 11 patient sera (64%) were found to contain elevated IL-10 levels (defined as >50% increase from controls). When correlating serum IL-10 levels with Fig. 5, we found a correlation between serum IL-10 levels and reversed inhibition by neutralizing antibodies. For instance, HN4 contained highly elevated IL-10 levels, and inhibition was reversed on the IL-10 assay. HN11, which also contained elevated IL-10 levels, also showed reversal of inhibition on neutralizing IL-10 assay. HN14 showed normal IL-10 levels on ELISA, but was reversed with neutralizing antibody. Finally, HN15, which was not reversed on IL-10 assay, also did not contain elevated IL-10 levels on our ELISA. Unfortunately, we were unable to evaluate serum IL-10 levels on HN12 due to inadequate supplies of serum. Only HN14 was not consistent with our thesis that the suppressive effect is mediated by IL-10. Overall, our data suggest that IL-10 plays an important role in mediating the immunosuppressive effect, although it probably does not act as the lone mediator.

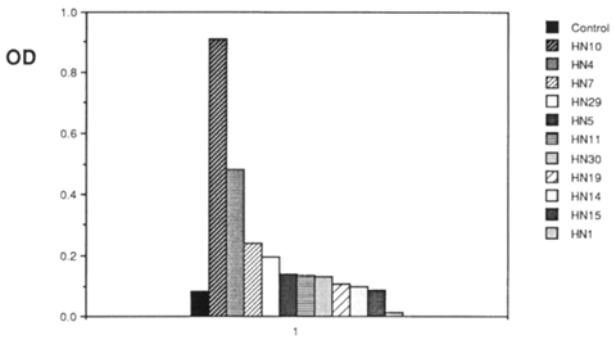


FIG. 6. IL-10 ELISA. Controls represent mean of three normal sera. Seven of 11 patient sera were found to contain elevated levels of IL-10. OD, optical density.

CONCLUSIONS

Numerous investigators have explored the role that IL-10 may play in inducing immunosuppression in patients with cancer. This cytokine has been isolated from the serum of patients with non-Hodgkins lymphoma and has been found to play a role as a prognostic indicator as well as in monitoring disease activity (35,36). In addition, it has been isolated from numerous human carcinoma cell lines, including those of the kidney, colon, breast, and pancreas; it also has been found in malignant melanomas and neuroblastomas (37). In a recent article, immunohistochemical staining of bronchogenic carcinoma (both adenocarcinomas and squamous cell carcinomas) demonstrated production of IL-10 by tumor cells (38). Finally, it has been demonstrated in high levels in basal cell carcinomas (39).

Our data show that TGF-β, a well-known immunosuppressive cytokine, is not responsible for the serum-induced immunosuppression seen in patients with squamous cell carcinoma of the head and neck. In addition, we have shown that the immunosuppresive effect is mediated before activation of T cells into T blasts. Once T cells are fully activated, the serum does not induce any suppressive effects. These data also suggest that a soluble IL-2 receptor is not competing for IL-2 and mediating the suppressive effect. Finally, in four of five experiments we were able to reverse the suppressive effect by blocking the biologic effect of IL-10. We have been able to show a correlation between serum IL-10 lev-

els and inhibition with a neutralizing antibody. This suggests that IL-10 acts either directly, or as a second messenger, in mediating the serum-induced immunosuppression.

Future studies will further examine the role of IL-10 and other TH2 cytokines (IL-4, IL-6) in mediating the serum-induced inhibition. In addition, we plan on determining IL-10 production by T cells after exposure to the serum factor. By removing macrophages and B cells from the incubation flasks, we will be able to determine which cell type is producing IL-10. Ultimately, a better understanding of host–tumor immune reactions will improve future use of immunotherapeutic regimens in patients with squamous cell carcinoma of the head and neck.

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