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Survey of immunosuppressive acidic protein and other immunological parameters in head and neck cancer patients

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Abstract

Serum levels of immunosuppressive acidic protein (IAP) and other immunological parameters were examined in 95 head and neck cancer patients and 27 control patients. The mean values of IAP in patients in the advanced stage were significantly higher than in early stage patients. Statistically significant increases in the mean concentration of IAP were also observed in patients with a recurrence, as compared to findings in those in the advanced stages. The mean values of blastogenesis response to PHA and NK cell activity in the cancer patients were lower than in disease free individuals, but with no statistical differences. In the end-stage patients, the IAP concentration was considerably elevated and the blastogenesis response showed a statistically significant decrease. Thus, the monitoring of serum IAP, in combination with other immunological parameters, aids in planning and assessing clinical staging in head and neck cancer patients.

Introduction

Patients bearing a malignant tumour may show systemic as well as local immune responses. Immune functions associated with neoplasia have been investigated using various specific or non-specific immunological parameters. Substances present in sera of patients with a malignancy, for example carcinoembryonic antigen (CEA; Gold and Freedman, 1965) in gastrointestinal tract cancer or alpha-fetoprotein (AFP; Okuda *et al.*, 1980) in hepatic cancer are specific marker proteins.

Hanna *et al.* (1990) reviewed serum tumour markers in case of head and neck cancer including CEA, ferritin, squamous cell carcinoma antigen and described their nature, the sources, uses and limitations. Although most markers lack a high degree of specificity and sensitivity, the combined measurement of more than one tumour marker may enhance diagnostic or therapeutic accuracy in head and neck cancer patients. Cellular immunity by natural killer cells or tumour infiltrating lymphocytes also play an important role in case of head and neck cancer (Schantz *et al.*, 1987; Snyderman *et al.*, 1989).

Immunosuppressive acidic protein (IAP) is an acute-phase tumour specific protein first detected in ascitic fluid of patients with stomach cancer (Tamura *et al.*, 1981; Sawada *et al.*, 1983). This alpha 1-acid glycoprotein is predominantly elevated in the sera of gynaecological (Sawada *et al.*, 1984), lung (Castelii *et al.*, 1989) and other malignant tumours (Kikuchi *et al.*, 1987; Miki *et al.*, 1987). IAP concentrations can now be readily determined using a sensitive assay method.

In the present report, we directed attention to serum IAP concentrations as well as other non-specific cellular immune functions in patients with head and neck cancers. The usefulness of combination assays of the immunological parameters during the course of treatment is also given attention.

Materials and methods

Patients

The group studied consisted of 95 Japanese patients with head and neck cancer (32 oral cavity cancers, 29 pharyngeal cancers, 16 laryngeal cancers, 16 maxillary sinus cancers and two others) and 27 control patients. The histological diagnosis was squamous cell carcinoma, in all these patients. The control group included adult patients with histologically benign disease such as adenoma of the thyroid gland. The primary tumour sites and tumour stages are listed in Table I (determined according to the UICC, TNM classification of Malignant Tumours, 1987).

Blood samples were assayed for immunological parameters within a few hours of acquisition. Most often, the blood samples were examined before and after the treatment and, in patients in the advanced stage, tests were repeated every third or fourth week during hospitalization.

Immunosuppressive acidic protein (IAP)

Serum IAP levels were measured by single radial immunodiffusion (Mancini *et al.*, 1965) using a commercial assay kit provided by Kayaku Antibiotics Research Co. (Tokyo, Japan).

Briefly, agarose gel containing rabbit anti-IAP serum was prepared on a glass plate with 2.5 mm-diameter wells. Five μl of serum samples were put into each well and the preparation was incubated at 37 °C in a humidi-

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TABLE I
SITES AND CLINICAL STAGINGS OF HEAD AND NECK CANCER PATIENTS

	Stage					
	I	II	III	IV	R	Total
Larynx	2	5	4	0	5	16
Pharynx	2	3	3	19	2	29
Oral Cavity	3	3	7	10	9	32
Maxilla	0	1	9	3	3	16
Others	0	0	0	1	1	2
Total	7	12	23	33	20	95

R: recurrence.

fied atmosphere. Each sample was tested in duplicate and the diameter of the precipitin ring was measured 48 h later.

A standard curve was based on purified IAP samples at the concentration of 250 and 1,000 μg/ml. Following tests done to many normal individuals from healthy volunteers and results reported elsewhere, an IAP value of less than 500 μg/mol was considered normal (Sawada *et al.*, 1983; Kikuchi *et al.*, 1987; Tanaka *et al.*, 1988).

Preparation of peripheral blood mononuclear cells (PBMC)

Following the centrifugation of peripheral blood on lymphocyte separation medium (LSM: Organon Teknika Corp., NC), the interface mononuclear cells were collected and suspended at a cell density of 5×10^5/ml in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10 per cent fetal calf serum (FCS). They were then subjected to further tests concerning lymphocyte blastogenesis, cytotoxity and analysis to determine the surface phenotype (Okamura *et al.*, 1988).

Lymphocyte blastogenesis

The lymphocyte blastogenesis stimulated with phytohemagglutinin (PHA-P, Difco) for three days were reported previously (Itoh *et al.*, 1988). Briefly, 5×10^5 PBMC in culture medium supplemented with or without 0.125 per cent PHA-P were incubated for 72 h, and ratio of resulted DNA contents were expressed as percentage (PHA-stimulated/unstimulated ×100). The mean value of PHA stimulation index (per cent) derived from normal individuals is set at 321±67, in our laboratory.

Cytolytic activities

The NK activities against NK-sensitive K-562 cells were assayed as previously reported (Yoda *et al.*, 1982). K-562 cell line cells were provided by the Foundation for the Promotion of Cancer Research (Tokyo, Japan). The cytolytic activity of fresh PBMC was assayed by a ^{51}Cr release assay for 4 h. PBMC (1×10^5) were added to wells of round-bottomed plastic 96-well microplates (Corning Glass Works) containing ^{51}Cr-labelled target cells (10^4) in 0.2 ml of RPMI 1640 medium supplemented with 10 per cent FCS and incubated for 4 h at 37 °C in humidified air containing 5 per cent CO_2. Thus, the effector cell/target cell ratio was 10. After incubation, the culture supernatants were harvested with the Skatron Titertek System (Skatron A.S., Lierbyen, Norway) and their radioactivities were determined by a gamma counter. The percentage of cytotoxity was calculated as follows:

$$\text{per cent cytotocixity} = 100 \times \frac{\text{experimental cpm—spontaneous cpm}}{\text{total cpm—spontaneous cpm}}$$

The spontaneous release observed with different target cells had a range of less than 10 per cent of the total lysis. The mean value of NK cell activity at our laboratory is designated at 30.8±10.0 per cent lysis.

Surface phenotype of culture cells

Flow cytometry analysis of cell surface phenotypes of fresh PBMC was carried out using a FACScan (Becton Dickinson, Mountain View, Calif. USA). Fluorescence data were collected by using logarithmic amplification on 10^4 viable cells as determined by both forward light scatter intensity and propidium iodide exclusion and the gated lymphocytes population were analyzed with staining patterns of the following monoclonal antibodies (Okamura *et al.*, 1989). The monoclonal antibodies used to determine the surface antigen profiles included Leu-5b/CD2, Leu-4/CD3, Leu-3a/CD4, Leu-2a/CD8 and Leu-12/CD19 purchased from Becton Dickinson. The mean percent value of each surface marker, as assessed in normal individuals in our laboratory was 79.4±7.3 for CD2, 67.9±9.6 for CD3, 42.2±8.9 for CD4, 26.7±6.9 for CD8 and 7.8±4.6 for CD19.

Statistics

Comparisons between groups regarding specific para-

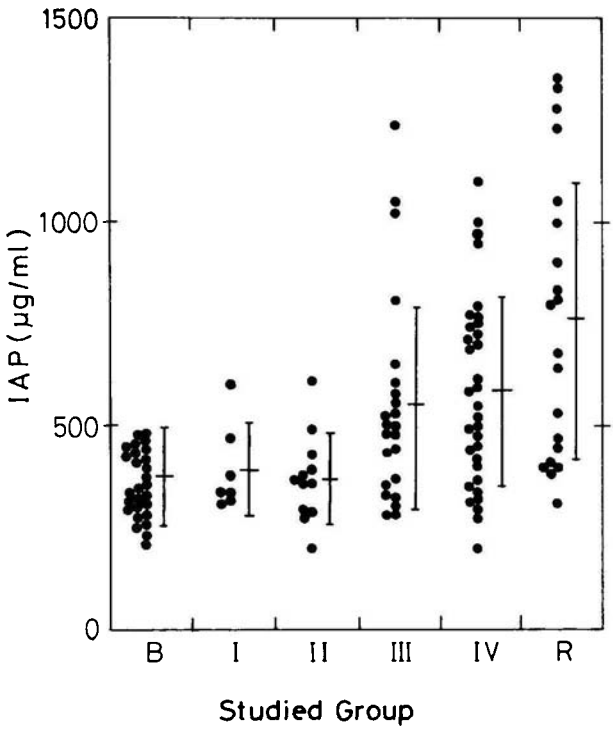

FIG. 1

Serum levels of IAP in benign controls (B), early (I, II), advanced (III, IV) stage and recurrent (R) head and neck cancer patients. Values are mean ±standard deviation (SD).

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TABLE II

MEAN VALUES OF SERUM LEVEL OF IAP, BLASTOGENESIS RESPONSE TO PHA, NK CELL ACTIVITY AND LYMPHOCYTE SUBSETS IN HEAD AND NECK CANCER AND CONTROL PATIENTS

	Patients					
	Stage I(7)	II(12)	III(23)	IV(33)	R(20)	B(28)
IAP (μm/ml)	391±110	371±108	554±255	578±278	770±349	377±119
PHA S.I. (%)	210±88	207±79	231±101	202±84	166±57	282±259
NK (% lysis)	17±13	25±28	13±7	17±11	14±10	19±13
CD3 (%)	64±12	66±8	66±10	65±10	57±16	NE
CD19 (%)	11±8	12±6	12±7	11±7	12±8	NE
CD2 (%)	83±5	83±5	78±8	77±12	74±11	NE
CD4 (%)	43±9	42±8	45±9	46±11	33±14	NE
CD8 (%)	25±13	29±9	23±7	25±9	28±12	NE

R: recurrence; B: benign disease; NE: not examined; ( ): number of patients examined.

meters were performed using Student's t-test. A p value of less than 0.05 was considered to have statistical significance.

Results

Overall comparison of immunological parameters

Results of comparisons between each stage of head and neck cancer patients and healthy controls for IAP, functional cellular immunity and lymphocyte subsets within peripheral blood are summarized in Table II. The mean level of serum IAP in the control group was 377±119 [mean ± standard deviation (SD)]. According to our criteria set at 500 μg/ml or less, as the normal concentration of IAP, none of the control group had abnormal values (Fig. 1).

Among 95 head and neck squamous cell cancer patients, serum IAP was elevated in one (14 per cent) of seven stage I patients, one (8 per cent) of 12 stage II patients, 11 (48 per cent) of 23 stage III patients, 19 (58 per cent) of 33 stage IV patients and 13 (65 per cent) of 20 patients of with a recurrence (Fig. 1), over-all positive rate being 47 per cent. The mean IAP values in the advanced stages (stages III and IV) were significantly higher than the early stages ($p<0.01$). Statistically significant increases in mean IAP values were also evident in recurrence group, as compared to findings in those in the advanced stages.

The average of lymphocyte blastogenesis response to PHA in cancer patients was lower than the normal range (set in our laboratory) in sera taken from disease free individuals. As the mean value of the PHA stimulation index (S.I.) varied in each group (including the 27 control patients), no significant diference could be obtained. There were no significant differences in NK cell activity or surface markers analyses for lymphocyte subsets in the studied groups.

The immunological parameters were checked every third or fourth week during hospitalization. The results of parameters in the final blood sample taken from a

TABLE III

IMMUNOLOGICAL PARAMETERS IN HEAD AND NECK CANCER PATIENTS WHO DIED SOON AFTER THE TEST

No.	Organ	Age	Gender	IAP	PHA	NK	CD3	CD19	CD2	CD4	CD8	CD4/CD8
1	Pharynx	53	m	2098	107	3.6	55.4	31.2	66.4	18.6	39.9	0.47
2	Pharynx	61	m	791	115	1.6	im	im	im	im	im	im
3	Parotid	47	m	1127	175	1.8	76.2	8.5	81.7	40.7	32.0	1.27
4	Pharynx	55	m	1047	im	im	69.8	2.1	86.3	12.3	62.1	0.20
5	Maxilla	69	m	956	142	2.7	68.4	1.7	87.3	42.2	25.9	1.63
6	Maxilla	73	m	426	im	1.0	57.4	3.9	84.1	26.7	44.1	0.61
7	Maxilla	70	m	459	130	14.6	52.5	8.5	86.5	40.1	22.5	1.78
8	Oral C.	64	m	527	im	7.8	66.9	4.3	90.7	23.4	38.4	0.61
9	Pharynx	63	m	1278	137	6.6	53.2	3.3	86.3	19.8	33.3	0.59
10	Larynx	61	m	1154	136	3.0	im	im	im	im	im	im
11	Pharynx	65	m	1661	104	11.1	58.6	9.2	71.0	28.0	27.3	1.03
12	Oral C.	68	m	725	161	12.9	60.8	0.7	82.1	36.9	21.1	1.75
13	Oesophagus	70	m	810	156	2.2	54.4	7.2	81.3	34.8	30.2	1.15
14	Maxilla	57	f	1110	121	6.1	32.1	2.3	73.3	19.3	18.0	1.07
15	Pharynx	41	m	1471	114	9.8	68.9	3.9	81.0	18.5	41.5	0.45
16	Pharynx	61	m	879	im	6.0	20.9	11.9	69.7	20.4	20.6	0.99
17	Larynx	64	m	1339	137	16.6	28.2	3.9	66.4	8.7	36.9	0.24
18	Larynx	45	m	1099	127	22.7	40.0	23.2	53.6	23.8	28.3	0.84
19	Pharynx	52	m	1946	114	11.8	30.1	3.3	79.6	14.6	13.5	1.08
20	Oral C.	38	f	1123	165	13.4	59.2	10.0	81.8	25.7	36.4	0.71
21	Oral C.	63	m	1207	180	5.3	77.9	3.2	81.0	45.3	31.4	1.44
22	Oral C.	69	m	1150	98	40.8	36.8	5.1	84.9	16.4	36.4	0.45
23	Oral C.	68	f	222	163	13.3	44.4	7.6	54.7	20.7	16.4	1.26
24	Pharynx	72	f	810	120	1.7	53.2	4.1	84.0	34.5	26.6	1.30
Average				1058	135	9.4	53.0	7.2	77.9	26.0	31.0	0.95
SD				450	24	8.9	16.0	7.4	10.2	10.5	10.9	0.47

im: Immeasurable due to insufficient numbers of lymphocytes.

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patient who died of the disease soon after the test are summarized (Table II) and compared with each stage of cancer patients (Fig. 2). The level of IAP was lower than the cut-off point (500 μg/ml) in only three patients, and the average of IAP was 1,058±450 μg/ml, a value significantly higher than that even in the recurrence group ($p<0.05$).

Lymphocyte blastogenesis response to PHA also decreased to 135±24 with a statistically significant abnormality compared with other stages or recurrences ($p<0.05$). These end-stage patients also had a low level of NK cell activity, but the difference did not reach statistical significance ($p = 0.05$). Percent values of lymphocyte subsets in the peripheral blood varied with the individual. It is of interest that average of CD8 was higher than CD4 (Fig. 2) and the CD4/CD8 ratio was significantly lower than in other stage patients ($P<0.05$).

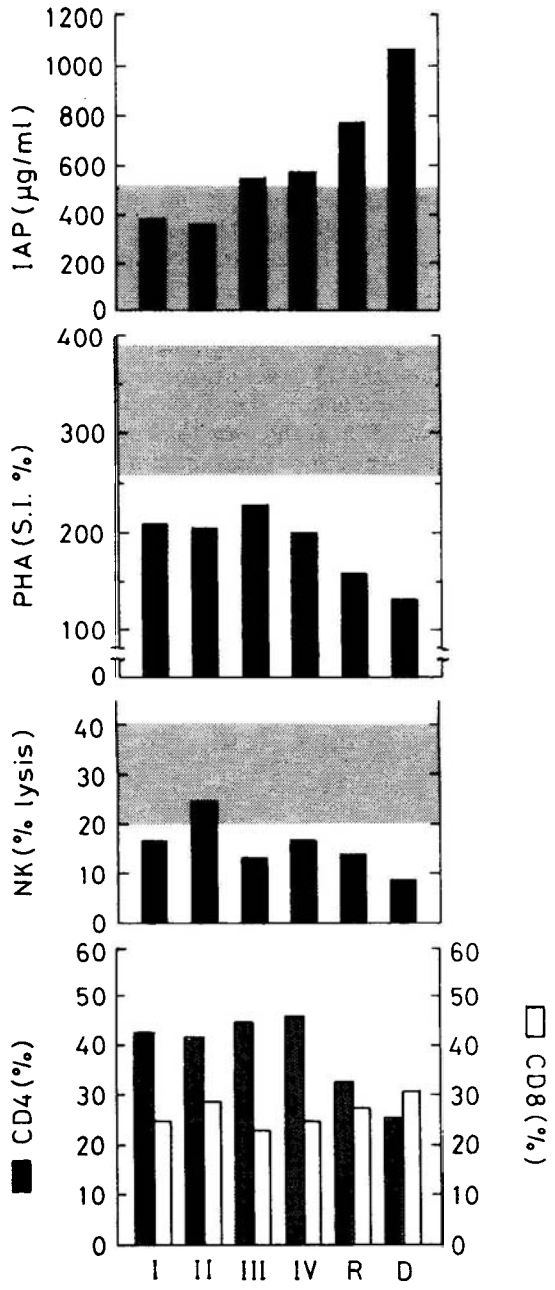

FIG. 2

Mean values of serum IAP, blastogenesis response to PHA, NK cell activity and lymphocyte subsets (CD4, CD8) in patients of stages I, II, III, IV, recurrence (R) and end-stage (D). Averages of end-stage are obtained from results in patients who died of disease soon after the test. Shadow areas in IAP, PHA (S.I.) and NK show the range obtained from normal individuals.

Changes in serum IAP before and after the treatment

For 32 patients with abnormal concentration of serum IAP before surgery or radiotherapy, the changes are shown as given in Fig. 3. In 16 patients (stage III five, stage IV nine, recurrence two) who were surgically treated, the concentration of IAP decreased in 14 and increased in two patients (stage III, hypopharyngeal cancer, 555 → 608 μg/ml; stage IV, mesopharyngeal cancer, 616 → 800 μg/ml). Eight of 14 patients with a decrease in IAP and one out of two with an increase in IAP died.

In 16 patients (stage II one, stage III three, stage IV five, recurrence seven) who received radiotherapy, the concentration of IAP decreased in 12 patients and increased in four (stage III, maxillary cancer, 950→1,440 μg/ml; stage IV, maxillary cancer, 737→1,262 μg/ml; stage IV, mesopharyngeal cancer, 546→1,407 μg/ml; recurrence, laryngeal cancer, 904→1,097 μg/ml). Seven of 12 patients with a decrease in IAP and two out of four with an increase in IAP died.

Case presentation (Figs. 4 & 5)

A 60-year-old Japanese man was admitted to our hospital for treatment of a metastatic skin lesion (Fig. 4a). He had undergone a pharyngo-laryngectomy and radiotherapy for a hypopharyngeal cancer ($T_3N_{2b}M_0$, stage IV) 14 months before. Although the lymphocyte blastogenesis response to PHA was lower then average, IAP and NK cell activity were normal and the CD4/CD8 ratio was 1.32 (Fig. 5). Radiotherapy to the neck was given from the fifth hospital week and the metastatic skin lesion at the right upper neck almost disappeared. However, other metastatic skin lesions at the lower neck appeared on the thirteenth week (Fig. 4b). Chemotherapy consisting of cisplatin (80 mg/m^2), peplomycin

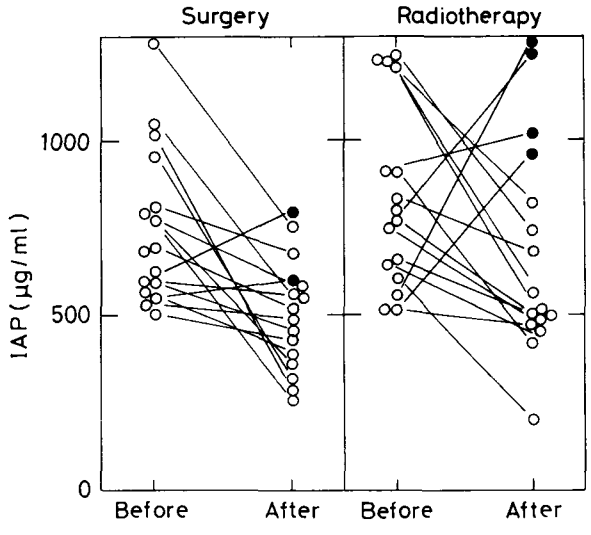

FIG. 3

Changes of serum levels of IAP before and after surgery or radiotherapy. Elevation of IAP even after the treatment is indicated by closed circles (●).

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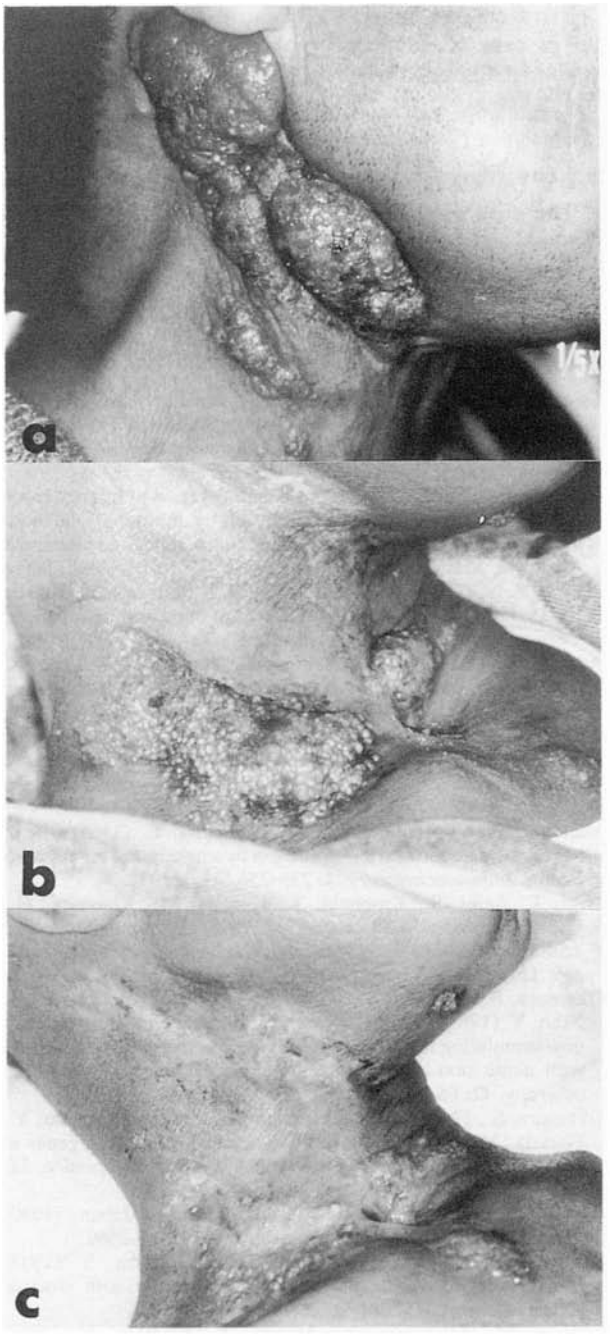

FIG. 4

Photographs of the metastatic skin lesions of a 60-year-old man. (a) Metastatic skin lesion on the right upper neck. (b) Photograph of the neck at the thirteenth hospital week. The subauricular metastatic skin lesions disappeared after radiotherapy to the neck. However, another metastasis appeared at the lower (supraclavicular) region. (c) Photograph of the neck after four courses of combination chemotherapy. The metastatic skin lesions have almost disappeared leaving a scar-like surface.

(40 mg/week) and methotrexate (40 mg/m^2) was initiated in the twelfth week. The IAP that had reached 707 μg/ml on the thirteenth week again decreased. Blastogenesis response to PHA could not be evaluated due to insufficient numbers of lymphocytes that became measurable in the sixteenth week. NK cell activity was 7.3, a value considerably lower than the average of 30.8.

Chemotherapy was given every fourth week. IAP that re-elevated right after the chemotherapy decreased again and fluctuated around 500 μg/ml. Other immunological parameters also fluctuated during the treatment. The metastatic skin lesion almost disappeared after the fourth course of chemotherapy (Fig. 4c). The patient was discharged from hospital and was prescribed additional mild chemotherapy (carboplatin 350 mg/m^2), every fifth week. At present he is doing well and leading a routine life.

Discussion

As the concentration of IAP was elevated in the sera of patients with advanced stages (III, IV) or in those with a recurrence, it can serve as a marker of tumour progression in head and neck squamous cell carcinoma patients. The significance of IAP as a marker of malignancy has been noted for various types of tumours. By comparing the serum levels of CEA, squamous cell antigen (SCC) and IAP in lung cancer and non-cancer pulmonary diseases, Castelli *et al.* (1989) observed the highest specificity for SCC and the highest sensitivity for IAP. Although the best accuracy was obtained with the combined determination of CEA and SCC, IAP was the most helpful in monitoring the acute phase reactions that frequently occur in case of lung cancers.

Sawada and colleagues (1984) reported the elevation of serum IAP in 62 per cent of gynaecological cancers as compared to 7 per cent of benign tumours. In the gynaecological cancers, the high concentrations of IAP were noted in case of 91 per cent of ovarian and 43 per cent of cervical cancers. In the ovarian cancer patients, elevation of IAP was observed at the early stage of the disease. Although they found no clear relationship between IAP values and histological types in that study, the usefulness of this protein in validating the histological predominancy was suggested. In comparison to evidence of the utility of IAP as a diagnostic and follow-up marker for various types of cancer, there is little documentation of this protein in case of head and neck cancers. Yamanaka *et al.* (1988) analyzed the values of IAP as well as another immunosuppressive substance (IS) in patients with head and neck cancer. In their study, the overall positive rate of IAP in 108 head and

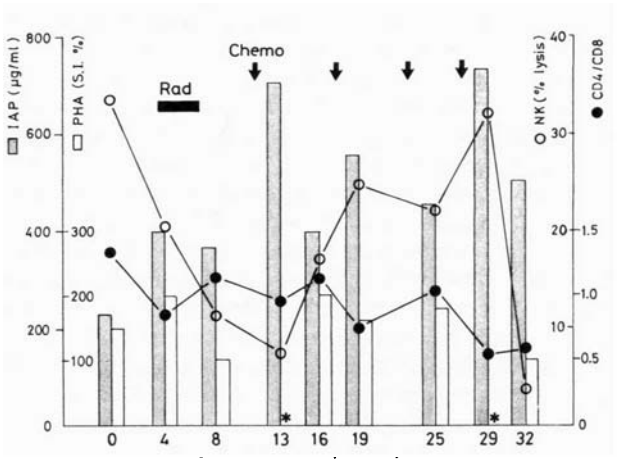

FIG. 5

Graph showing serial serum levels of IAP, PHA S.I. NK cell activity and CD4/CD8 ratio in the same patient as in Fig. 4. Radiotherapy and chemotherapy (consisting of cisplatin, peplomycin, methotrexate) were given as indicated. Blastogenesis response to PHA was not measurable due to insufficient numbers of lymphocytes on thirteenth and twenty-ninth week (*).

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neck patients was 37 per cent respectively, values significantly higher than in the controls. In laryngeal carcinomas, the concentration of IAP was elevated according with advance of the disease.

This elevation was also noted for other types of cancer. Significantly different elevations of IAP observed in advanced or recurrent head and neck cancer patients in our study also support the usefulness of this protein for monitoring the disease stage. Marked elevation of IAP in the sera obtained from end-stage patients who died of disease soon after the test indicate that IAP is actually an acute phase marker in the late stage of the disease. It was interesting that the serum IAP normalized or at least decreased than before surgery or radiotherapy was given. The concentration of IAP temporarily elevated following the chemotherapy again decreased. Thus, the concentration of IAP has potential for use in evaluating the effects of treatment in head and neck cancer patients. It may also aid in determining whether the treatment of a tumour is successful or likely to end in a recurrence.

In comparison to the results of IAP values that show a significant elevation according to stage of the disease, a survey of changes of other parameters failed to show significant differences between each stage. We should recognize, however, there was evidence that mean values of other parameters such as PHA S.I. or NK cell activity of the examined cancer patients were considerably lower than the normal range in disease-free individuals.

The correlation of the level of IAP and other immunological parameters needs further investigation. The immunosuppressive acidic protein was observed to suppress both phytohaemagglutinin-induced lymphocyte blast formation and the mixed-lymphocyte reaction *in vitro* (Tamura *et al.*, 1981; Shibata *et al.*, 1983). In tumour bearing patients, inhibition of lymphocytes function by IAP was also suggested (Kikuchi *et al.*, 1987).

The marked decrease of blastogenesis response to PHA and NK cell activity in patients in the advanced stage of disease and, particularly, in end-stage patients in our study indicates that cellular immunity is suppressed by IAP in head and neck cancer patients.

It was of interest that the CD4/CD8 ratio was reversed in the end-stage patients. Snyderman *et al.* (1989) analyzed phenotypic variations of lymphocytes infiltrating head and neck tumours. Using flow cytometric analysis of tumour infiltrating lymphocyte suspensions, they observed a significant decrease in CD8 cells ($p<0.05$) in patients with cervical metastasis. As a result, the CD4/CD8 ratio greater than one may be a useful prognostic indicator of cervical metastasis. Wolf *et al.* (1987) found that the mean helper/suppressor cell ratio (CD4/CD8) in peripheral blood samples increased progressively with increase in the tumour stage. Conversely, Tancini *et al.* (1990) analyzed T lymphocyte subsets in case of early and metastatic solid tumours and observed a significant decrease in the CD4/CD8 ratio in those with a metastatic cancer. They also suggested the decline of T helper subpopulations in most patients with tumour dissemination. As the CD4/CD8 ratio became less than one only in the end-stage patients in whom IAP was remarkably elevated in our study, there probably is an alteration in immune functions by the suppressive effect by IAP.

In conclusion, serum IAP determinations may be useful in case of advanced or recurrent head and neck cancer patients to monitor the results of treatment or clinical staging.

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