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The primary immune response of patients with different stages of squamous-cell bronchial carcinoma

H M JANSEN,[2] T H THE,[1] G C DE GAST,[1] M T ESSELINK,[1] G PASTOOR,[1] AND N G M ORIE[2]

From the Clinical Immunology Unit[1] and the Pulmonology Division,[2] Department of Medicine, University of Groningen, the Netherlands

Jansen, H M, The, T H, de Gast, G C, Esselink, M T, Pastoor, G, and Orie, N G M (1978). *Thorax*, **33**, 755–760. **The primary immune response of patients with different stages of squamous-cell bronchial carcinoma.** Using the indirect ELISA technique, the IgM, IgG, and IgA antibody response to the primary test immunogen *Helix pomatia* haemocyanin (HPH) was studied in 30 patients with various clinical stages of primary squamous-cell bronchial carcinoma and compared with values obtained in 15 controls matched for sex, age, smoking habit, and presence of chronic bronchitis. Patients with disseminated disease (stage III) showed a significant decrease in IgG and IgA antibody response ($P<0.001$), but IgM antibodies were relatively high and not different from the controls. Although normal IgG and IgA antibody titres were found at the peak response two weeks after immunisation in patients with localised disease (stage I), these antibody titres showed a significantly more rapid decline after serial investigations at eight and 14 weeks after immunisation compared with the controls ($P<0.001$) despite total removal of the tumour burden at *c* four weeks after immunisation. In-vitro HPH-induced lymphocyte transformation was considerably decreased in stage I patients ($P<0.01$) as well as in stage III patients ($P<0.001$). The results suggest that patients with squamous-cell bronchial carcinoma develop impaired T-cell function, which gives rise to a defective antibody response and in-vitro lymphocyte reactivity to the T-cell dependent primary immunogen HPH.

Many attempts have been made to investigate the immune responsiveness in relation to the development and course of cancer in animal tumour models and in man (Hellström and Hellström, 1974; Herberman, 1974; Price-Evans, 1976). In these studies an in-vitro test, such as antigen-induced lymphocyte transformation reaction, can be used for T-lymphocyte function. The possible importance of the role of T-lymphocytes in the humoral immune response acting on B-lymphocytes producing antibodies against thymus dependent antigens is less often studied in tumour bearing individuals. Our purpose was to investigate the class-specific humoral immune response of patients with bronchial carcinoma in relation to the specific cellular immune reactivity after immunisation with a primary test antigen.

It is generally agreed that the test antigen used in these studies is important in assessing the results. Among others (VX 174) haemocyanin from *Helix pomatia* can be used to test the primary, thymus dependent, antibody response (De Gast *et al*, 1973). *Helix pomatia* haemocyanin (HPH) is a macromolecular respiratory protein from the Roman snail (*Helix pomatia*), which can easily be isolated and has known physical and chemical properties. It can be used as a non-toxic strong primary immunogen. The assessment of class-specific antibody titres to HPH in healthy adults was recently studied in our laboratory, using the enzyme-linked immunosorbent assay (ELISA) (Weits *et al*, 1978). This ELISA technique is a sensitive and relatively simple quantitative test (Engvall and Perlmann, 1972; *Bulletin World Health Organisation*, 1976; Ruitenbeek *et al*, 1976). We investigated the primary HPH-induced IgM, IgG, and IgA antibody response in patients with squamous-cell bronchial carcinoma in re-

lation to the in-vitro HPH-induced lymphocyte stimulation. Results were related to the clinical stage and subsequent course of the disease.

Methods

Thirty patients with histologically confirmed, primary, squamous-cell bronchial carcinoma (mean age: 64·8, range 52–77) classified in TNM stages according to the criteria for lung carcinoma proposed by the International Union Against Cancer (UICC, Geneva, 1974) were studied. In addition a control group of 15 individuals with chronic bronchitis matched on age, sex, and smoking habit and without evidence of malignant disease was studied. None received chemotherapy, radiotherapy, or corticosteroids before or at the time the tests were performed.

IMMUNOLOGICAL STUDIES

HPH was provided by the biochemical department of the University of Groningen and used in purified form as described by De Gast *et al* (1973).

Patients were immunised when the diagnosis was established (before any treatment was started) with 1 mg HPH subcutaneously in the deltoid region as described by De Gast *et al* (1973).

Serum for antibody titre determination was collected before and two, eight, and 14 weeks after immunisation. Two weeks after immunisation venous heparinised blood was taken for lymphocyte stimulation tests. The same serial studies were done in the control group. Stage I patients (localised disease) underwent surgery between two and eight weeks after immunisation. In most of the stage III patients (disseminated disease) full serial studies were impossible because of intercurrent adjuvant palliative chemotherapy or radiotherapy.

LYMPHOCYTE STIMULATION TEST

Lymphocyte cultures were performed according to a microculture technique in round-bottom Cooke(R) microtitre plates as described by Du Bois *et al* (1974). HPH-induced lymphocyte stimulation was recorded in 100×10^3 lymphocytes/well in the presence of 25% normal human pool serum. The sera were inactivated for 30 min at 56°C and stored at −80°C. Two weeks after immunisation lymphocytes were cultured in the presence and absence of 50 μg HPH for five days and incubated at 37°C in humidified 5% CO_2 in air. On the fourth day, 25 μl 6−^{3}H thymidine (Radiochemical Centre, Amersham, England, 0·5 μCi: specific activity 400 mCi/mM) was added and incubated for 16 h. Harvesting was done on the fifth day with a multiple cell culture harvester (Skatron, Norway) using glassfibre filters. The filters were dried at 60°C for 60 min and transferred to counting vials. After the addition of 5 ml of scintillation fluid, counting was performed in a liquid scintillation counter (Packard Tricash-2450).

The following formula was used to determine the disintegrations per minute (dpm) per mm^3 of peripheral blood

$$\frac{\text{dpm/well in stimulated cultures} - \text{dpm/well in unstimulated cultures}}{\text{number of ly/well}} \times$$

number of ly/mm^3 peripheral blood.

DIFFERENTIAL LEUCOCYTE COUNTS

Quantitative differential leucocyte counts were performed using the spin-smear method to avoid unequal distribution of the different cell types in the spreads on the glass slides. We consider the counted leucocytes as a random sample of those in the smear and make the extent of the inaccuracy that is inevitably inherent in the method used for differential counts as small as possible (Rümke *et al*, 1975). Two hundred cells were counted twice in different randomised chosen fields. The mean value of the counted percentages of lymphocytes was used.

ANTIBODY DETERMINATION

The method of antibody determination was described by Weits *et al* (1978). The indirect ELISA technique was performed in round-bottom microtitre plates (Greiner, M 24-AR). Coating with antigen was obtained with 0·1 mg HPH in 0·1 ml 0·1 M NaHCO3 with 0·02% sodiumazide at pH 9·6 during incubation overnight at 4°C. The plates were washed with tap water and 0·1 ml aliquots of doubling serum dilution starting with 1 : 10 were then added. After washing 0·1 ml of 1 : 5000 diluted, monospecific rabbit antihuman IgG, IgM (Central Bloodtransfusion Service, Amsterdam, batch numbers KH 15-17-AO1 and KH 16-113-A1) or IgA antiserum (prepared in our own laboratory) was added and incubated for 45 min at 37°C. After washing 0·1 ml, 1 : 2500 diluted, peroxidase labelled sheep anti-rabbit antiserum (Institut Pasteur, Paris, batch no 09-7502), diluted in PBS 0·01 M+2% BSA, was added. Incubation was performed at 37°C for 45 min. After the last washing 0·1 ml of a 5-aminosalicylic acid 0·08% and hydrogen-peroxidase 0·05% solution, 9 : 1, pH 6·00 were allowed to react for one hour, after which time either the results were read immediately or the reaction was stopped by adding 10 μl

N NaOH. The highest dilution with a clearly brown colour was taken as a titre. A positive and negative serum pool were always included in each series of measurements as a standard. The negative pool always had a titre <1 : 40, or the series was discarded. The positive pool had a titre of 1 : 1280, and only small titre variations were allowed and corrected for. The results are expressed in terms of reciprocal of the ^{2}log of the highest dilution of each serum that resulted in a positive reaction (1 : 20 dilution taken as 0).

SERUM IMMUNOGLOBULIN (IG) CONCENTRATIONS

Serum concentrations of IgM, IgG, and IgA were determined by the automated immunoprecipitin method (Dr Th Ockhuizen, Immunochemistry Laboratory, Department of Internal Medicine).

The fluoronephelometric determination of the antigen-antibody complexes was performed on the Technicon continuous-flow automatic analyser, according to the instructions of the manufacturer (Technicon method no SE4-0038 FE5). Reagents and antisera were supplied by Technicon. Mean serum immunoglobulin concentrations of healthy individuals were: IgM: 104 (±2 SD: 45–235); IgG: 1187 (±2 SD: 800–1760); and IgA: 168 (±2 SD: 90–315).

STATISTICAL ANALYSIS

The results obtained were analysed by the Student *t* test. P values less than 0·05 were considered significant.

Results

Data on age, sex, clinical stage and lymphocyte stimulation by HPH two weeks after immunisation, anti-HPH antibody titres two weeks after immunisation, and serum Ig concentrations of the patients and controls are summarised in the table.

ANTIBODY RESPONSES, LYMPHOCYTE STIMULATION, AND CLINICAL STAGE

As can be seen from fig 1 (upper panel) the primary anti-HPH class-specific responses two weeks after immunisation of patients with localised disease (stage I) (mean ^{2}log titre IgM, 10·09±0·97 SD; IgA 11·09±0·95; IgG 11·23±1·47) were not different from the controls (IgM 10·09±1·17; IgA 11·03±0·82; IgG 10·36±1·31). In patients with disseminated disease (stage III) IgA (8·62±1·54) and IgG (7·88±2·04) antibody titres were significantly lower than the controls (respectively $P<0·001$ and $P<0·001$). In this latter group the IgM antibody titre (9·42±1·49) did not differ from the controls. Figure 1 (lower panel) illustrates the results of HPH-induced lymphocyte stimulation tests in the different stages of the disease compared with the controls. Results in stage I (mean: 36·93 ±27·79 dpm/mm^3) and in stage III (mean: 8·64±9·03 dpm/mm^3) were significantly lower than in the controls (mean: 294·62±251·91) (respectively $P<0·01$ and $P<0·001$).

ANTIBODY RESPONSES, SERIAL STUDIES

In 10 patients with localised disease and in 14 control subjects serial ELISA tests could be performed on sera collected eight and 14 weeks after immunisation.

Figure 2 shows graphically that 14 weeks after immunisation in stage I patients the mean percentage IgA antibody titre decline (24·76±3·31 SEM) and IgG antibody decline (24·85±3·75 SEM) is statistically significant, more pronounced than in the controls (respectively 18·69±2·31 SEM and 12·69±2·9 SEM) ($P<0·001$). There was no differ-

Mean values of antibody response and in-vitro lymphocyte stimulation to HPH, differential leucocytes counts, and serum immunoglobulin concentrations in patients with squamous-cell bronchial carcinoma and in chronic bronchitic controls. (Figures in parentheses=ISD)*

No	Age	Sex	^{2}log antibody titre 2 weeks after immunisation			In-vitro lymphocyte-stimulation			Peripheral blood		Serum immunoglobulin concentrations		
			IgM	IgA	IgG	No antigen	HPH	HPH/mm^3	ly%	leuco/mm^3	IgM	IgA	IgG
Patients (stage I)													
15	62·9 (5·6)	15m	10·09 (0·97)	11·09 (0·95)	11·23 (1·47)	482·3 (264·5)	2194·5 (1422·7)	36·9 (27·8)	26·9 (8·7)	8533·3 (1625·1)	96·8 (24·6)	216·7 (51·1)	1497·3 (271·5)
Patients (stage III)													
15	66·2 (8·4)	14m 1f	9·42 (1·49)	8·62 (1·54)	7·88 (2·04)	289·3 (116·0)	825·9 (639·9)	8·6 (9·0)	21·8 (8·8)	8433·3 (3429·6)	99·1 (27·9)	295·0 (121·9)	1386·0 (330·5)
Controls													
15	60·5 (8·0)	14m 1f	10·09 (1·17)	11·03 (0·82)	10·36 (1·31)	840·9 (845·1)	1 3092·4 (9210·8)	294·6 (251·9)	37·6 (9·8)	6453·3 (1346·9)	102·7 (37·5)	266·7 (80·3)	1278·7 (232·7)

*Individual results can be obtained from the author.

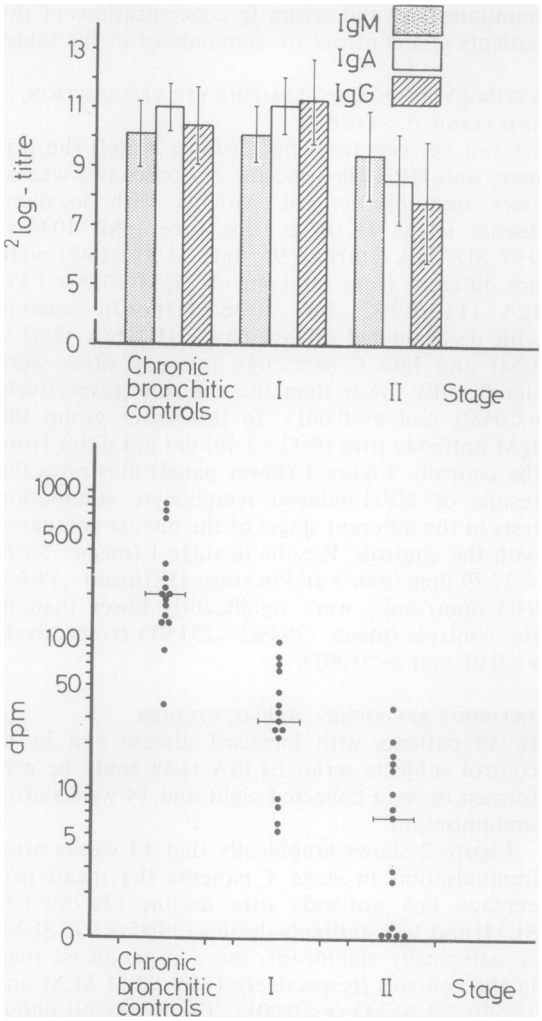

Fig 1 *Relation between class-specific antibody response (expressed as mean ^{2}log titre±SD) and in-vitro lymphocyte stimulation (expressed as dpm/mm^3, cross bars indicate median scores) to HPH in 30 patients in various clinical stages of squamous-cell bronchial carcinoma, compared with chronic bronchitic controls. Antibody titres and lymphocyte stimulation were determined two weeks after immunisation. Patients in stage III had significantly decreased IgG and IgA antibody titres (P<0·001). Patients in stages I and III had significantly lower lymphocyte stimulation scores (respectively P<0·01 and P<0·001).*

ence in decline of the IgM antibody titre found in patients (20·59±5·89 SEM) compared with controls (18·23±4·08 SEM) within that period.

Serum IgG, IgA, and IgM concentrations were within the normal range in the patients as well as in the controls (see table).

Discussion

It is generally agreed that cellular immune mechanisms are affected in the host defence against cancer. Humoral factors, however, may also play an important part. In animal models tumours have been found to inhibit antibody synthesis (Kamo *et al*, 1957). Mitchell *et al* (1976) have reported that in tumour-bearing mice both antigen and antibody were necessary to produce inhibition of "armed" cytotoxic cells through mechanisms that appear to affect an intermediary thymus-dependent suppressor cell. The same sort of antibody-induced mechanisms were suggested by Gershon *et al* (1974).

This study of immune responsiveness to HPH of patients with squamous-cell bronchial carcinoma showed changes in the humoral and cellular response in different stages of the disease. The results were compared with values obtained in a matched control group because it is important to select such a group by factors that can probably influence immunocapacity as well as the development and occurence of lung cancer (Wal *et al*, 1966; Cohen *et al*, 1977).

The changes in the humoral immune response two weeks after immunisation consisted of a decreased class-specific IgG and IgA antibody response in patients with disseminated disease (stage III) in combination with a relatively high IgM anti-HPH antibody response. Although normal IgG and IgA anti-HPH antibody titres were found at the peak response two weeks after immunisation in patients with localised disease (stage I), a more rapid decline in these antibody titres was shown at eight and 14 weeks after immunisation. These differences in titre decline between patients and controls occurred despite apparent total removal of the tumour, and we consider they were not caused by surgical intervention as we have shown no such changes in an additional control group of seven patients who underwent thoracotomy for benign pulmonary disease (unpublished data).

Titre decline studies in stage III patients were not feasible because of the adjuvant chemotherapy and radiotherapy given in that group. Primary cellular immune capacity measured by in-vitro lymphocyte stimulation two weeks after HPH immunisation was significantly decreased, but this was more pronounced in stage III patients.

The findings obtained in the present study are partly in agreement with a previous study in melanoma patients, but the haemagglutination technique used in that study is a more sensitive test for 19S than for 7S antibodies (De Gast *et al*,

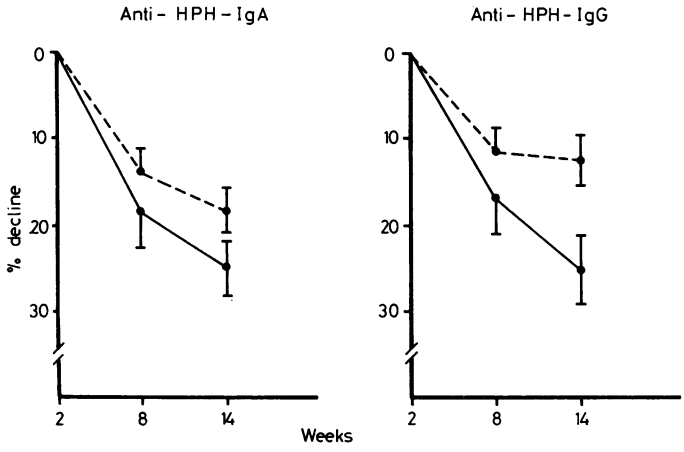

Fig 2 *Relation between antibody titre decline (expressed as mean percentage decline of values obtained at two weeks after immunisation ±SEM) of anti-HPH-IgA and anti-HPH-IgG titres, at eight and 14 weeks after immunisation in stage I patients (●———●) compared with the controls (●- - - -●). For both antibody titres there was a significantly more rapid decline in patients compared with the controls (respectively* $P<0.01$ *and* $P<0.001$*).*

1976). Using the indirect ELISA technique we found in stage III patients a dissociation of IgG and IgA from IgM anti-HPH antibody titres. The humoral immune response to thymus-dependent antigens requires a functioning T-cell system to serve merely as helper cells for B-lymphocytes, which provide the actual antibody formation (Anderson *et al*, 1970; Geha *et al*, 1974; Janossy *et al*, 1975; Herber-Katz and Wilson, 1976). HPH has such properties of a thymus-dependent antigen (De Gast *et al*, 1973; 1976; Weits *et al*, 1978).

Our studies suggest that defects in T-helper cell function may give rise to decreased thymus-dependent antibody formation. Since IgG and IgA antibodies are generally thymus-dependent and IgM antibodies more thymus-independent, our findings in the patients with disseminated disease may reflect a decreased T-helper function in these patients. The decreased HPH-induced lymphocyte stimulation found in our patients may be an additional argument for defective T-cell function, as sensitised T-cells seem to be necessary for antigen-induced lymphocyte stimulation (Geha *et al*, 1973).

On the other hand, it has recently been shown that in tumour-bearing animals reactive T-cells did recognise tumour cell immunogens as strong antigens but failed to co-operate with B-cells for an antibody response against the tumour cells (Dennert *et al*, 1977), which is necessary to "arm" the FC-receptor on T-cell subpopulations with these antibodies to direct their cytotoxic activities against tumour cells (Saal *et al*, 1977). There is evidence that this disturbance in T-cell function is a consequence of the activation of suppressor T-cells by the tumour (Reinisch *et al*, 1977). Moretta *et al* (1977) have recently examined the ability of two T-cell subpopulations with FC-receptors to provide help or to suppress polyclonal B-cell differentiation. They showed that suppressor T-cells after interaction with IgG immune complexes suppress the generation of plasma cells when added to helper T-cells. We have suggested in a previous study the existence of circulating IgG-immune complexes in patients with bronchial carcinoma (Jansen *et al*, 1977). The presence of these circulating complexes was related to the extent of tumour burden. So the diminished thymus-dependent antibody production in bronchial carcinoma patients may be an IgG-immune complex-induced hyperactivity of suppressor T-cell function leading to a more rapid decline of specific, thymus-dependent antibody formation and decreased HPH-induced lymphocyte stimulation, already demonstrable in stage I of the disease after removal of the tumour but still more pronounced in stage III.

In conclusion changes in the thymus-dependent primary humoral and cellular immune response were found in patients with squamous-cell bronchial carcinoma related to the extent of the disease. The results suggest that in these patients a decreased T-helper function exists, which is possibly a consequence of an immune complex activated T-suppressor cell.

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Requests for reprints to: Dr H M Jansen, Department of Pulmonology, State University Hospital, Oostersingel 59, Groningen.