Virchows Arch [Pathol Anat] (1986) 409:211–221

Virchows Archiv A

Immunocytochemical characterization of lymphocytes in benign and malignant lymphocyte – rich serous effusions*,**

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Summary. The cytological diagnosis of malignant Lymphoma in serous effusions can be difficult because reactive lymphocytes may be morphologically indistinguishable from malignant cells in lymphocytic and other low grade Non-Hodgkin's lymphomas. As a result of the present study, diagnostic accuracy can be improved by means of B- and T-cell enumeration using an immunoalkaline-phosphatase method (IAP). 30 cytological specimens, including 28 pleural, 1 pericardial and 1 ascitic fluids, were studied with a panel of monoclonal anti B- and anti T-cell antibodies (PAN B, kappa, lambda, T1, T2, OKT4, T8). Reactive lymphocytic effusions were characterized by a predominance of T cells constituting $\geq 80\%$ of all lymphocytes with an excess of helper/inducer cells (mean helper to suppressor ratio 3.0) and by a surface kappa to surface lambda ratio of 1.6 on B-cells. Tuberculous effusions showed a similar distribution of lymphocyte-subpopulations whilst most of the carcinomatous fluids showed a lower percentage of T cells (lowest value 67%) and lower Th:Ts ratio (mean 2.0). Lymphoid cells in samples of five B-cell lymphomas were characterized by T-cell depression ($\leq 70\%$). B-cells in three cases expressed clear cut light chain monoclonality which was at least suggested in the other two cases.

Lymphoid cells from two cases of Hodgkin's disease expressed an indistinct immunological pattern. Labelling of cytoplasmic immunoglobulins (heavy and light chains) using the peroxidase antiperoxidase method (PAP) may be important to characterize neoplasms of the plasma cell series.

It is concluded that the chosen panel of antibodies in combination with IAP labelling method may be of great value in identifying B-cell lymphomas. The technique can be used in the routine laboratory and storage of unlabelled and labelled slides over long periods is possible.

* Dedicated to Professor K. Lennert, Kiel, on the occasion of his 65th birthday

** This study was supported by the Krebsliga St. Gallen/Appenzell

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Key words: Immunocytochemistry – Cytopathology – Serous effusion – Malignant lymphoma – Reactive lymphocytosis

Introduction

The cytological diagnosis of serous effusions containing numerous lymphoid cells often presents difficulties. A method allowing differentiation between distinct benign conditions (e.g. non specific lymphocytosis vs. tuberculosis) as well as between stimulated lymphoid cells and lymphoid neoplasia would be of great value. In many cases this is not possible on cytomorphological basis only (Spieler 1979; Spriggs and Vanhegan 1981; Melamed 1963). Two recent studies have shown that some of these diagnostic problems can be elucidated with B- and T-cell enumeration using immunocytochemical techniques (Ghosh et al. 1985; Martin et al. 1984). In contrast with former procedures analyzing T and B cells – by rosetting with sheep red blood cells and immunofluorescent staining (Domagala et al. 1981; Krajewski et al. 1982; Manconi et al. 1978; Pettersson et al. 1978) – the immunocytochemical labelling technique can be applied to routinely performed cytological smears; laboratory procedure is simple, the material may be stored for long periods (Ghosh et al. 1985) and the labelled cells exhibit well preserved morphological details.

We have used an immunoalkaline phosphatase technique (IAP) for surface markers of T- and B-lymphocytes and a peroxidase antiperoxidase method for cytoplasmic immunoglobulins. Benign lymphocytic effusions have been compared according to their a etiology and with those containing malignant lymphomas.

Material and methods

Patients and laboratory procedure. 30 specimens were studied, obtained from 30 consecutive patients, submitted for routine cytological evaluation. They included 28 pleural, 1 pericardial and 1 ascitic fluid. The samples for the study had to be rich in well preserved cells with 80% or more lymphoid elements. Based upon the final diagnosis (cytomorphological criteria, clinical information and bacteriological results) the specimens were divided into six groups: 1) 8 patients with nonspecific, reactive lymphocytic effusions. 2) 3 patients with tuberculous pleurisy, where the diagnosis was based on positive culture for Mycobacterium tuberculosis in one and on a favorable response to tuberculostatic therapy in two others. 3) 6 patients with a lymphocytic effusion containing carcinoma cells: four suffered from breast cancer and two from pleura-infiltrating carcinoma, probably mesothelioma. 4) 6 patients with known carcinoma but without neoplastic cells in the effusion. 5) 5 patients with effusion positive (3), consistent (1) or suspicious (1) for Non-Hodgkin's lymphoma (NHL) of whom four have had histological confirmation of the malignant lymphoma. Histological typing according to the Kiel classification was centroblastic-centrocytic lymphoma (cb–cc) in three and Immunocytoma (IC) in one case. 6) 2 patients with known Hodgkin's disease presenting with effusion containing abundant atypical lymphocytes.

After centrifugation at 2,800 rpm for 10 min, the supernatant was decanted and three direct smears were prepared from the button for conventional cytological examination. The remaining cell material was washed at least three times with physiological saline and then evenly smeared. A minimum of 12 slides have been prepared. Half of the slides were fixed

Table 1. Primary monoclonal antibodies used for immunocytochemical characterization of lymphocytes

Antibody	Specifity against	Source
PAN B	B-cells	DAKO
kappa	B-cells	Dr. Odermatt
lambda	B-cells	Dr. Odermatt
T 1	T-cells	DAKO
T 2	T-cells	DAKO
OKT 4	T-helper/inducer cells	Ortho
T 8	T-suppressor/cytotoxic cells	DAKO

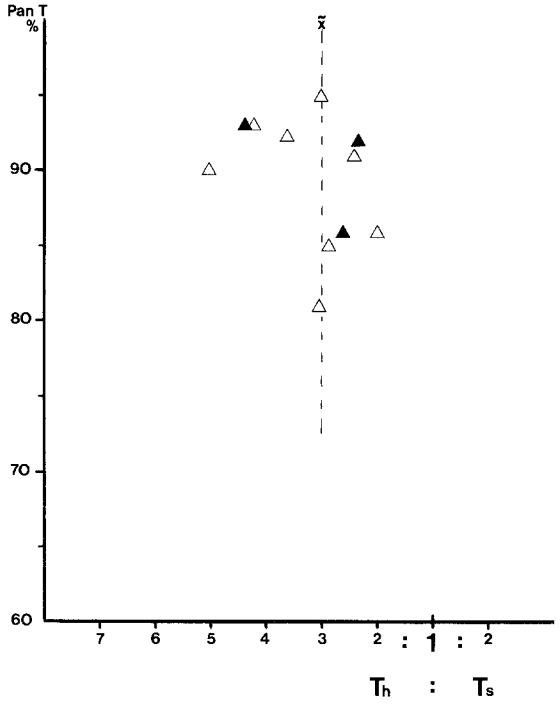

Fig. 1. Reactive and tuberculous lymphocytic fluids; enumeration of T-cells (percentage and *Th: Ts* ratio) (=▲ tuberculous pleurisy; △ nonspecific reactive lymphocytic effusion)

and stained according to the Papanicolaou method for subsequent intracytoplasmic immunoglobulin staining. The other half were air dried for May-Grünwald-Giemsa staining (1–2 smears) and for surface marker studies. In cases of delayed immunocytochemical procedure, air dried smears were stored at −20° C wrapped in aluminium foil. From blood rich specimens the mononuclear cells were isolated by density centrifugation using Lymphoprep®.

In each case the same panel of antibodies was applied for determination of T and B lymphocytes. Antibodies to T-cell markers PAN T, T helper/inducer (Th), T suppressor/cytotoxic (Ts) and to B-cell markers PAN B, IgA, IgM, IgG, kappa (cytoplasmic and surface), lambda (cytoplasmic and surface) and, as a control, to albumin were used. Immunocytochemical labelling was performed for each marker on a separate slide.

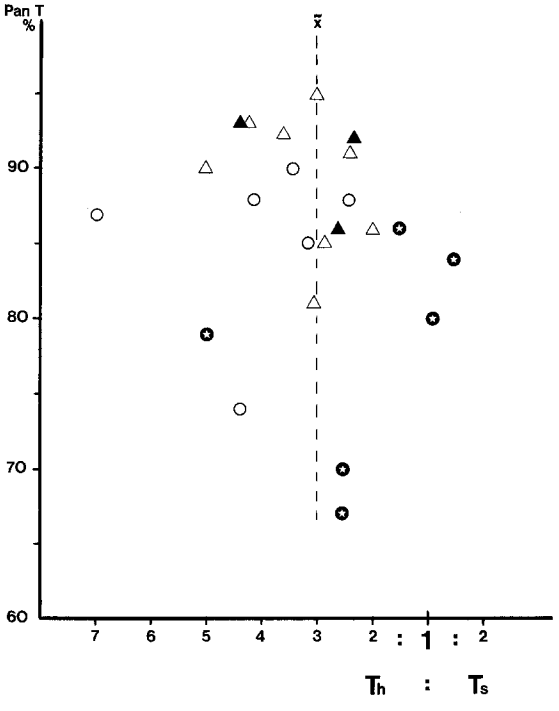

Fig. 2. All reactive non-lymphomatous fluids; enumeration of T-cells (percentage and *Th:Ts* ratio) (▲ = tuberculous pleurisy; △ = nonspecific reactive lymphocytic effusion; ○ = known cancer – no tumor cells in effusion; ✪ = lymphocytic effusion with carcinoma cells)

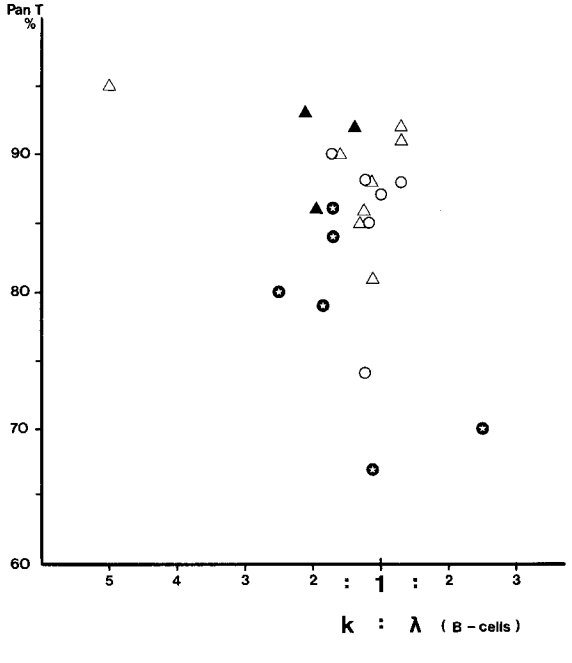

Fig. 3. All reactive non-lymphomatous fluids; percentage of T-cells and enumeration of B-cells (kappa:lambda ratio) (▲ = tuberculous pleurisy; △ = nonspecific reactive lymphocytic effusion; ○ = known cancer – no tumor cells in effusion; ✪ = lymphocytic effusion with carcinoma cells)

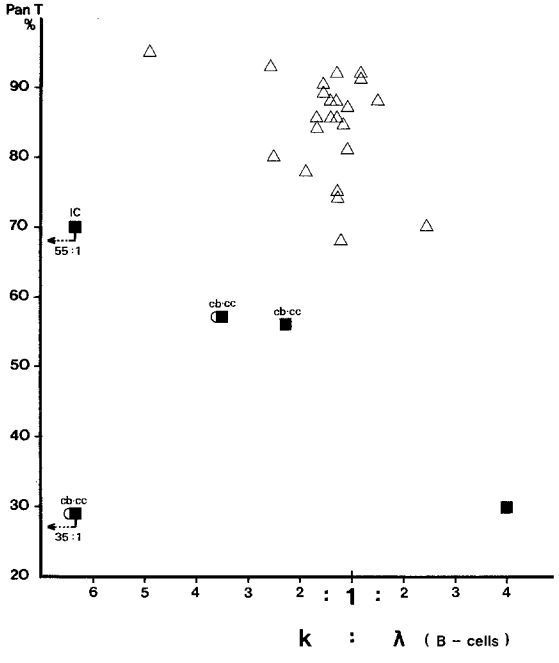

Fig. 4. Comparison of reactive and lymphomatous fluids according to T-cell percentage and B-cell enumeration (kappa:lambda ratio) (△ = non-lymphoid disease; ■ = NHL, B-type; [◪ = cytomorphology consistent/suspicious])

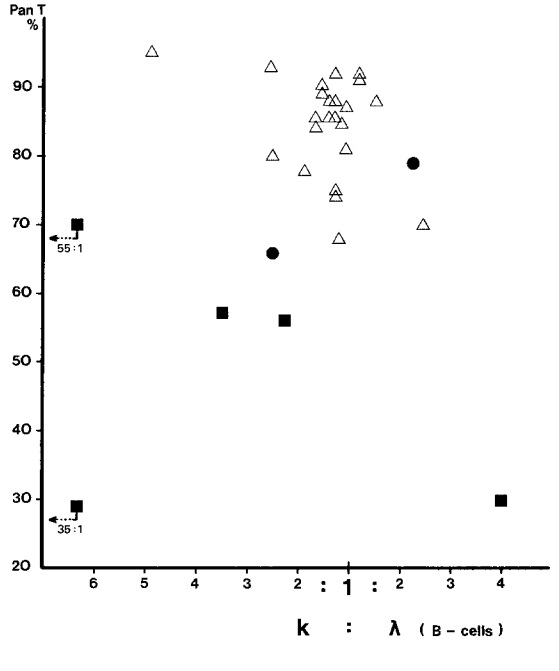

Fig. 5. Lymphocyte-rich effusions in Hodgkin's disease; comparison of T-cell percentage and B-cell enumeration with the results on fluids of lymphomatous (B-NHL) and reactive origin (△ = non-lymphoid disease; ■ = NHL, B-type; ● = lymphogranulomatosis Hodgkin)

Reagents. Polyclonal antisera against human IgA, IgG, IgM, kappa, lambda and albumin, swine anti rabbit IgG and the PAP-complex were purchased from DAKO. The primary monoclonal antibodies used for immunocytochemical characterization of lymphocytes are described in Table 1. Rabbit antiserum against mouse immunoglobulin was purchased from DAKO, alkaline phosphatase conjugated goat antiserum against rabbit IgG from Sigma.

Immunocytochemical staining procedure. Immunostaining for intracytoplasmic immunoglobulins using the PAP-method was performed on already fixed and Papanicolaou-stained smears by the same technique as used for paraffin-embedded histological sections published elsewhere (Schmid et al. 1982). Labelling for surface markers was performed by an immunoalkaline phosphatase method published and modified by Feller and Parwaresch (1983). The air dried smears were briefly fixed in acetone. Antisera were then applied in the following order: monoclonal primary antibody (PAN B, T1, T2, T8, OKT4, kappa and lambda), rabbit anti-mouse immunoglobulins and antirabbit IgG alkaline-phosphatase conjugated. Each incubation lasted 30 min and was followed by washing in Tris-buffer (pH 7.4). Alkaline phosphatase reactivity was demonstrated with fast-red TR salt (Sigma, St. Louis, MO, USA) and naphthol-AS-BJ-phosphatase (Sigma) as substrate, each dissolved in dimethylformamide (DMF) (Merck, Darmstadt, FRG) and diluted with 0.05 M propandiol buffer, pH 9.75. Endogenous alkaline phosphatase was inhibited by adding 1 mM levamisole (Sigma) to the incubation medium. The slides were incubated at room temperature for 10 min, washed in Tris buffer pH 7.4, counterstained with haemalaun and mounted in glycerine gelatin.

Data analysis. One hundred lymphoid cells were counted, independent of their morphology, on each slide labelled for surface T- and B-markers (T, Th, Ts, PAN B, kappa, lambda). The percentage of T-cells, calculated out of the sum of labelled B and T cells, and both Th:Ts-ratio and kappa: lambda-ratio were calculated. The results of these marker studies were compared concerning the six groups described above. The results of intracytoplasmic Immunoglobulin labelling (heavy and light chains) were estimated and determined as negative, weak, moderate or strong for the different cell types (plasmacytic cells and tumorcells).

Results

Labelling for cytoplasmic Ig produced an intense brownish red staining mostly all over the visible cytoplasm. On smears of non-malignant lymphocytic fluids plasmocytoid and mature plasma cells reacted with anti-Ig antibodies exhibiting a polyclonal pattern. As a rule these cells were only sparsely scattered throughout the smears. In many cases histiocytes and carcinoma cells stained weakly to strongly positive in a pattern suggesting polyclonality. In all these cases albumin control was positive too. Among the five B cell-lymphomas only the tumour cells from the histologically proven immunocytoma exhibited a positive monoclonal Ig staining, according to the known paraproteinaemia IgG/kappa. Albumin control was negative in tumour cells.

Figures 1–5 show the results of staining for surface T and B cell markers on lymphoid cells in smears of lymphocyte-rich serous fluids. The percentage of T cells and ratios (Th:Ts/kappa: lambda) were compared between the different groups defined above. Immunostaining produced an intense, focally granular red product with distribution all over the cell surface (Fig. 6, 7). Histiocytes and granulopoietic cells also stained positive with variable intensity, probably due to incompletely inhibited endogenous alkaline phosphatase. Yet they could easily be distinguished from lymphoid cells on morphological grounds.

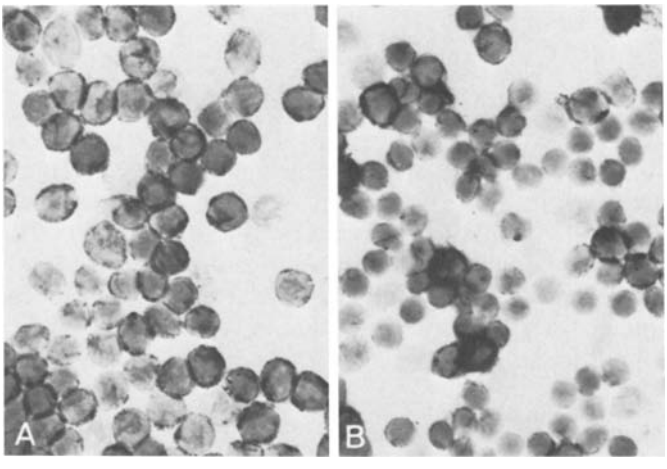

Fig. 6A and B. Reactive lymphocytic fluid (MB Nr. 4635/85). Ratio Th:Ts = 2.6 **A** Staining for T_h cells and **B** staining for T_s cells. More cells with a granular product over the cell surface in **A** than in **B**. (IAP staining for surface markers, × 800)

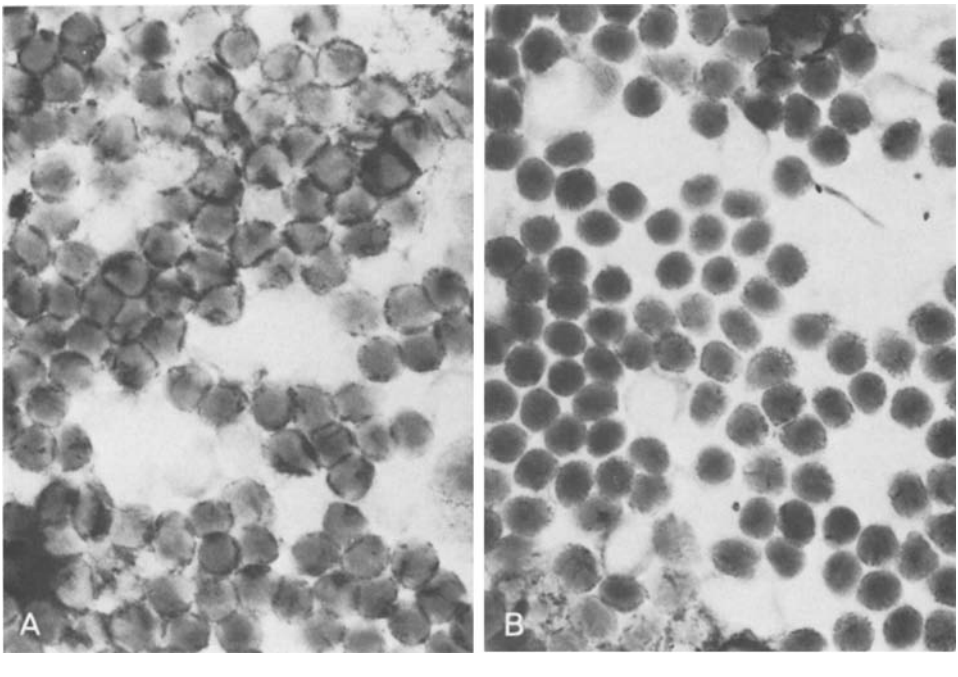

Fig. 7A and B. Non-Hodgkin-lymphoma, monoclonal kappa (histological diagnosis centroblastic-centrocytic m.L.) (MB Nr. 19538/85). Ratio kappa: lambda = 35.0. **A** Staining for kappa light chains and **B** staining for lambda light chains: Strong positivity of most of the cells in **A** and only occasional positive cells in **B**. (IAP staining for surface markers, × 800)

Reactive and tuberculous lymphocytic fluids
(Figs. 1, 2 and 3)

Most of the lymphocytes reacted with the monoclonal anti-T cell antibodies. The percentage of T-cells (T2) was over 80% in all cases (mean 89.5%). The staining for Th and Ts cells revealed a marked preponderance of T helper/inducer cells with a ratio Th:Ts from 1.9 through 5.1 (mean 3.0) (Fig. 6). Less than 20% of the lymphocytes reacted with monoclonal anti-B cell antibodies (PAN B). The kappa:lambda ratio ranged from 5.0 through 0.8 (mean 1.6). There were no differences in the results of marker studies on nonspecific lymphoid effusions and on fluids due to tuberculosis.

Known carcinoma, effusions without tumourcells
(Figs. 2 and 3)

The percentage of T-cells and Th:Ts ratio and kappa: lambda ratio of B-cells ranged within the same values evaluated for nonspecific and tuberculous effusions in four cases. In one case a slightly lower percentage of T-cells (74%) and in an other case a marked excess of T helper-cells (Th:Ts = 7.5) was observed.

Known carcinoma, effusions with tumourcells
(Figs. 2 and 3)

In four cases the cancer cells were consistent with known breast carcinoma, in one case with malignant mesothelioma and in one case with adenocarcinoma of unknown origin. Distinct differences were observed when comparing results of lymphocyte markers with those of the two groups described above. Three of the six cases showed quantitative T-cell values below 80% (79%, 70%, 67%). Mean ratio Th:Ts was 2.0, two cases revealing reverse ratios, 0.9 and 0.7.

Only one case did not match the kappa:lambda ratio expressed in non carcinomatous reactive fluids, showing an excess of B-lambda cells (kappa: lambda = 1:2.5).

Non-Hodgkin lymphomas
(Fig. 4)

Of the five B-cell lymphomas, four were histologically proven. Each case revealed a percentage of T-lymphocytes ≤ 70 (70, 57, 56, 30, 29%). Three of the five cases expressed monoclonality of either kappa light chains (kappa:lambda = 35.0; 55.0) (Fig. 2) or lambda light chains (kappa:lambda = 0.25). The two other cases showed a tendency to monoclonality with kappa: lambda ratio 3.5 and 2.4.

Hodgkin's disease
(Fig. 5)

Samples of the two cases contained numerous atypical lymphoid cells which expressed T2 in 79% and 66% respectively. No diagnostic cells of Hodgkin/Sternberg type could be observed. kappa:lambda-ratio was very different at 0.4 and 2.5 respectively.

Discussion

The diagnosis of involvement of body cavity fluids with lymphoid malignancies on purely cytological variables can be extremely difficult particularly in lymphomas of low and intermediate grade (lymphocytic (CLL), centrocytic, centroblastic-centrocytic, lymphoplasmocytoid type) (Martin et al. 1984; Melamed 1963; Spriggs and Vanhegan 1981). This fact prompted us to evaluate surface markers on lymphoid cells independent of their morphology. The results of this study, although based on small number of fluids, show that immunocytochemical staining for surface markers is strikingly helpful in the diagnosis of B-cell lymphoma.

Previous studies (Domagala et al. 1981; Krajewski et al. 1982; Pettersson et al. 1978) have used the rosetting test with sheep red blood cells to identify T-cells and immunofluorescent staining with antisera against heavy and light chains to enumerate B-cells. For the present study two labelling techniques were selected, which several authors have shown to yield accurate results offering evident advantages (Feller and Parwaresch 1983; Ghosh et al. 1983; Ghosh et al. 1985; Martin et al. 1984; Walts and Said 1983). Advantages of IAP- and PAP-labelling technique on cytological smears are as follows: cytoplasmic as well as surface antigens are expressed, cellular morphology is well preserved and can be matched with immunocytochemically expressed markers, immunostaining may be performed on smears even after storage over a long period, stained smears can be stored as record and for reevaluation, material for immunolabelling can be prepared as part of routine laboratory procedure. In comparison with the non-lymphomatous fluids, all cases of B-cell lymphoma showed a reduction of percentage of T cells ($\leq 70\%$), two cases revealing very low values. The increased B cells expressed light chain monoclonality in three cases and a tendency to monoclonality in two cases. The missed clear expression of monoclonality in two cases is suggested to be based on technical reasons (cell evaluation regardless of atypias) in combination with a mixed population of malignant and reactive lymphoid cells. Three of the four histologically proven lymphomas were low grade tumours of follicle centre cell-type (centroblastic-centrocytic). Tumour cells of the fourth case, classified as lymphoplasmocytoid immunocytoma (IC), expressed heavy chain monoclonality (for light chain staining was no material available) with an antibody against cytoplasmic Ig as well, consistent with the known paraproteinemia IgG kappa. In an other case of known CLL the abundant lymphoid cells in pleural effusion

were considered reactive; enumeration of lymphocyte surface markers fully approved the morphological diagnosis (T cells 87%, ratio kappa: lambda 1.2) and also did the clinical follow up.

Our results are comparable with those reported in previous studies using either immunofluorescence technique or immunocytochemical labelling techniques. For B-cell lymphomas a marked reduction in the percentage of T cells (Domagala et al. 1981; Krajewski et al. 1982) and a preponderance of B cells respectively (Ghosh et al. 1985) were reported. Further, monoclonal light chain immunoglobulin expression was observed in six of nine lymphomas by Krajewski et al. (1982), in 16 of 20 cases by Martin et al. (1984) and in two of four multiple myeloma by Walts and Said (1983).

In the two cases of Hodgkin's disease morphologically abnormal lymphoid cells, but no Hodgkin/Sternberg-Reed cells were present in effusions. Immunological studies yieled an indistinct pattern. For obvious reason (very small number of cases) further conclusions are not possible. Still, it should be considered, that atypical lymphoid cell infiltrates may either be due to tumourous involvement of serous membranes or to thoracal radiotherapy following mediastinal involvement by Hodgkin's disease.

The helper to suppressor ratio among T cells in reactive effusions, ranging from 5.1 to 1.9 (mean 3.0), is in keeping with previous investigations reporting mean values of 3.5 (Ghosh et al. 1985) and 6.0 (Martin et al. 1984). Further a clear predominance of percentage of T cells, mostly $>80\%$, was shown by several authors (Domagala et al. 1981; Ghosh et al. 1985; Krajewski et al. 1982; Pettersson et al. 1978). In tuberculous pleural effusions percentage of T cells, Th:Ts ratio and kappa:lambda ratio of B cells revealed no differences compared with nonspecific fluids, hence differentiation between tuberculous and non-tuberculous pleurisy is not possible based on immunocytochemical enumeration of lymphocytes. Still, Pettersson et al. (1978) reported an absolute number of T lymphocytes significantly higher in pleural fluid than in peripheral blood in patients with pulmonary tuberculosis.

The six lymphocytic, but not carcinomatous fluids accompaniing epithelial neoplasia showed equivocal differences in helper to suppressor values and kappa to lambda ratios compared with pure reactive effusions. On the other hand the ratio of helper/inducer to suppressor/cytotoxic T-cells in six carcinomatous effusions was in most cases lower than that found in reactive fluids; in two cases the ratios were even reverse. An interpretation of these findings is not possible because of the small number of cases. Further investigations with more cases may possibly disclose T-cell enumeration as prognostic factor for serous membrane-infiltrating carcinomas. In a few cases a PAN T-antibody staining all, especially peripheral, T-cells was applied (T1). The mean percentage of positive T-cells was only 5% higher than with antibody labelling to T2.

In many cases labelling with anti immunoglobulin antibodies and with antibodies against T- and B-cell markers yielded weakly to strongly positive stained granulocytes and histiocytic/mesothelial cells. As a rule the identification of non lymphoid cells gives no problem on morphological criteria. The positive cytochemical reaction may be due to endogenous alkaline phos-

phatase (IAP) and/or peroxidase (PAP) or to in vivo uptake and subsequent storing of immunoglobulins from the serous fluid (labelling of heavy and light chains). Same or similar mechanisms are supposed to be responsable for occasional staining of carcinoma cells. Endogenous formation of immunoglobulins in lymphomatous and carcinomatous cells will be confirmed by negative albumin labelling.

We believe enumeration of lymphocytes according to their surface marker-phenotype, determined by an immunoalkaline phosphatase procedure, to be a simple and reliable method which allows the differentiation between reactive and malignant lymphoid cell populations with a high probability. The technique can be used in the routine laboratory and storage of the slides before and after immunocytochemical procedure gives no problems.

Acknowledgements. We are indebted to PD Dr. A.C. Feller, Department of Pathology, University of Kiel, FRG for kindly supporting us with the IAP-technique. We also thank Dr. B. Odermatt, Department of Pathology, University of Zurich for generously providing kappa and lambda antibodies. We are grateful to the staff of the Cytology Laboratory, above all to M. Hell (CTA), for their technical help in preparing the material.

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Accepted December 12, 1985