J Clin Pathol 1985;**38**:608–612

Immunocytochemical staining of T and B lymphocytes in serous effusions

AK GHOSH, AI SPRIGGS,* DY MASON

*From the Nuffield Department of Pathology, John Radcliffe Hospital, Oxford, and the *Clinical Cytology Department, Churchill Hospital, Oxford*

SUMMARY Cell smears from serous effusions containing large numbers of lymphoid cells were stained by the alkaline phosphatase-anti-alkaline phosphatase technique with a panel of monoclonal antibodies, including anti-B and anti-T cell antibodies and anti-HLA-DR. Samples from 17 patients with lymphoproliferative disorders—such as chronic lymphocytic leukaemia and non-Hodgkin's lymphoma—and from 19 patients who had no evidence of lymphoid neoplasia—for example, cases of carcinoma, cardiac failure—were investigated. The majority of lymphoid cells in reactive effusions were T cells, which lacked HLA-DR and showed a marked excess of helper/inducer cells (mean helper to suppressor ratio of 3·5). In contrast, lymphoid cells in samples from nine cases of B cell neoplasia were positive for B cell antigen and HLA-DR. In a further four B cell neoplasms most lymphoid cells were reactive T cells. Two cases of T cell lymphoid leukaemia could also be characterised by immunocytochemical staining, both being classified as T helper cell neoplasms. Labelling was performed on routinely prepared, air dried cell smears, which could be stored in the unfixed state for long periods before staining. The technique may therefore be of use in many clinical cytology laboratories for the diagnosis of effusions containing numerous lymphoid cells.

The cytological diagnosis of serous effusions containing numerous lymphoid cells may present difficulties.[1 2] Previous authors have suggested that T and B lymphocyte enumeration may be useful in the diagnosis of such cases.[3–5] In these studies it was first necessary to separate mononuclear cells from effusion fluids and then to analyse them in suspension for T cell markers (rosetting with sheep red blood cells or response to phytohaemagglutinin stimulation) or B cell markers (immunofluorescent staining for surface immunoglobulin). This approach, however, not only requires a fresh sample but also entails the inconvenience of having to perform labelling within a few hours.

We have used an immunoalkaline phosphatase labelling technique which can detect T and B cell markers in routinely prepared, air dried smears from serous fluids, even after storage of smears for long periods. We have established the patterns seen in reactive lymphocytic effusions and compared the results with those seen in samples from patients with lymphoid neoplasia.

Material and methods

PATIENTS AND SAMPLES

Thirty six pleural or peritoneal effusion samples which had been sent to the Clinical Cytology Department of the Churchill Hospital, Oxford, were used in this study. These samples comprised a control group of lymphocytic effusions from 19 patients with non-lymphoid malignancy or with benign conditions (such as cardiac failure) and effusions from 17 patients with lymphoid neoplasms. Clinical diagnosis in the latter group had been based on haematological examination of blood and bone marrow samples or on results of surgical biopsy. A number of these cases had also been typed immunologically by staining smears or peripheral blood cells, as described previously.[6]

Samples were collected into universal containers containing edetic acid as anticoagulant. Smears of cell deposit were prepared for conventional cytological examination and stained with May-Grünwald-Giemsa and Papanicolaou stains. In addition, cells from some samples were washed once with 5% bovine serum albumin and then smeared. Spare unstained air dried smears were stored at −20°C (wrapped in aluminium foil) for subsequent immunocytochemical staining. Smears which had been stored for up to five years at −20°C were included.

ANTIBODIES

Details of the monoclonal antibodies used in this study are shown in Table 1. Sheep antiserum against mouse immunoglobulin was prepared in the author's

Accepted for publication 23 January 1985

Table 1 *Monoclonal antibodies used for immunocytochemical staining of serous effusions*

Antibody	*Specificity*	*Source*
UCHT1	Anti-T cell	Dr PCL Beverley
DAKO-Pan B	Anti-B cell	Dakopatts a/s
CR3/43	Anti-HLA-DR	Author's laboratory
T3-10	Anti-T helper cells	Dr G Riethmuller
DAKO-T8	Anti-T suppressor cells	Dakopatts a/s

laboratory (DYM) by a conventional immunisation schedule. Monoclonal anti-alkaline phosphatase was prepared as described previously and used to prepare alkaline phosphatase-anti-alkaline phosphatase (APAAP) complexes.[7]

IMMUNOCYTOCHEMICAL TECHNIQUE

Fixation of smears

Frozen smears were warmed to room temperature before being unwrapped. Smears were then fixed for 10 min in acetone:methanol (1:1) and transferred to 0·2 M Tris buffered saline, pH 7·6.

Staining

Labelling was performed by the immunoalkaline phosphatase procedure as described previously.[7,8] Briefly, fixed smears were incubated in turn with monoclonal antibody, unlabelled sheep anti-mouse immunoglobulin, and finally with APAAP immune complexes. In most experiments additional incubations with the anti-mouse immunoglobulin and APAAP complexes were performed after the first three steps to enhance the intensity of the reaction.[7] Slides were washed briefly in Tris buffered saline between each incubation step. The alkaline phosphatase reaction product was visualised using fast red and naphthol AS-MX phosphate as substrate.[8] Endogenous alkaline phosphatase was inhibited by the addition of 1 mM levamisole to the substrate solution.

Results

Tables 2 and 3 show the results of staining for T and B cell markers on smears of serous effusions. Positive cells stained an intense red and there was no background labelling of antigen negative lymphoid cells or red blood cells (Figs. 1–3). There was no difference in staining between washed and unwashed cells. Although macrophages are HLA-DR positive, they could easily be distinguished from lymphoid cells on morphological grounds (Fig. 1a).

NON-LYMPHOID DISEASE (Table 2)

Most of the lymphocytes in these samples reacted with the monoclonal anti-T cell antibody (mean 85·2%). The numbers of lymphocytes reacting with the monoclonal anti-B cell antibody and with anti-HLA-DR were similar and usually represented less than 20% of all lymphoid cells. In 11 samples (from which spare smears were available) staining was performed for T helper/inducer and T suppressor/cytotoxic cells. The sum of these T cell subsets approximated to the total number of T cells. These samples all showed a marked excess of T helper/inducer cells (mean helper to suppressor ratio of 3·5) (Figs. 1b and c).

CHRONIC LYMPHOCYTIC LEUKAEMIA AND LYMPHOMA (Table 3)

One case of B cell chronic lymphocytic leukaemia (patient 20) contained almost 100% B cells. These were further typed and found to be IgM and λ positive (in agreement with studies performed on

Table 2 *Immunocytochemical labelling of serous effusions from patients with non-lymphoid disorders*

Patient no	*Diagnosis*	*T cells*	*B cells*	*HLA-DR*	*Th*	*Ts*	*Th/Ts*	*Interpretation*
1	Cardiac failure	77	24	23	NT	NT	NT	Reactive T cells
2	Cardiac failure	87	6	13	NT	NT	NT	Reactive T cells
3	Recurrent pneumothorax	92	10	15	NT	NT	NT	Reactive T cells
4	Post-pneumonic	89	9	16	67	19	3·5	Reactive T cells
5	Rheumatoid arthritis	92	8	12	60	28	2·1	Reactive T cells
6	Tuberculosis	85	10	8	57	23	2·5	Reactive T cells
7	Tuberculosis	90	9	6	65	31	2·1	Reactive T cells
8	Tuberculosis	83	13	20	63	22	2·9	Reactive T cells
9	Angioimmunoblastic lymphadenopathy	76	24	23	62	17	3·6	Reactive T cells
10	Melanoma	82	6	7	NT	NT	NT	Reactive T cells
11	Carcinoma of breast	82	11	16	70	20	3·5	Reactive T cells
12	Carcinoma of lung	78	11	13	60	17	3·6	Reactive T cells
13	Carcinoma of lung	90	7	10	72	20	3·6	Reactive T cells
14	Carcinoma of lung	83	15	13	61	17	3·6	Reactive T cells
15	Carcinoma of lung	86	12	10	77	20	4·0	Reactive T cells
16	Mesothelioma	82	NT	12	NT	NT	NT	Reactive T cells
17	Carcinomatosis	89	10	12	NT	NT	NT	Reactive T cells
18	Unknown	86	9	13	NT	NT	NT	Reactive T cells
19	Unknown	90	4	12	NT	NT	NT	Reactive T cells
	Mean	85·2	11	13·4	64·9	21·2	3·5	

All values represent percentage positive lymphoid cells.
Th = helper/inducer T cells, Ts = suppressor/cytotoxic T cells.
NT = not tested.

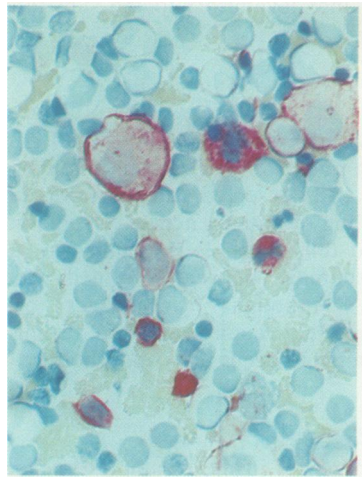

Fig. 1a

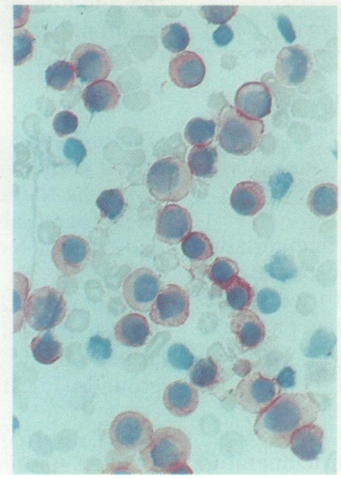

Fig. 1b

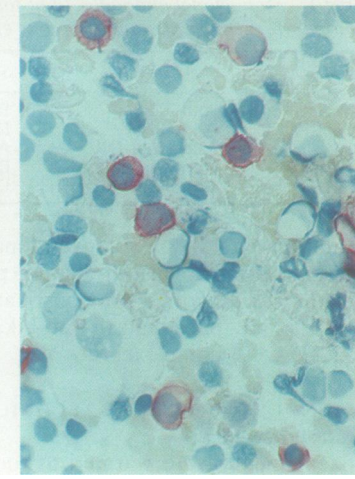

Fig. 1c

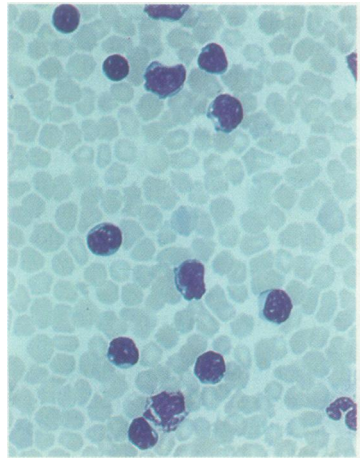

Fig. 2a

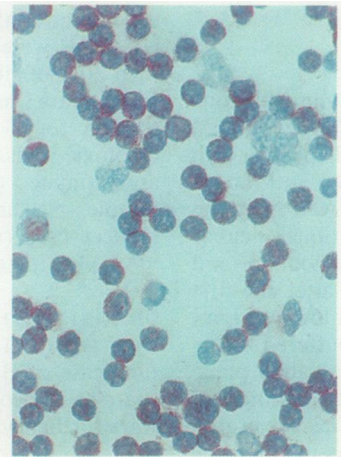

Fig. 2b

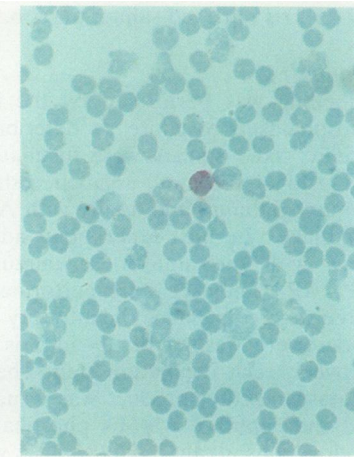

Fig. 2c

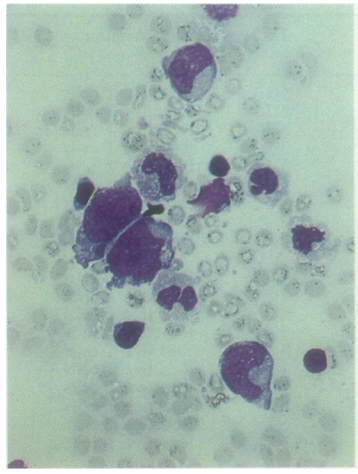

Fig. 3a

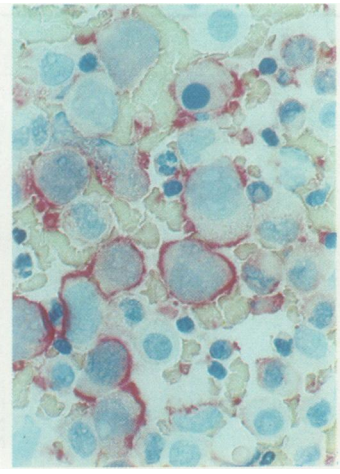

Fig. 3b

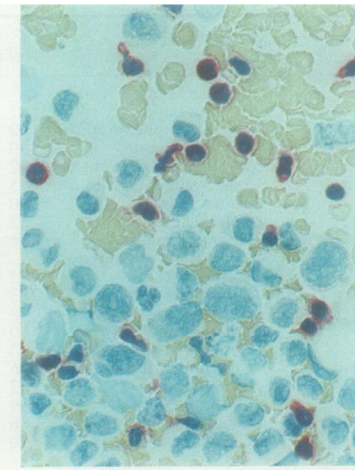

Fig. 3c

Fig. 1 *Reactive lymphoid pleural effusion from a patient with carcinoma of the lung (patient 13). (a) A few normal B lymphocytes and macrophages express HLA-DR. (b) Numerous T helper/inducer cells are present. (c) T cytotoxic/suppressor cells are seen in lower numbers.*

Fig. 2 *Smears of a pleural effusion from a patient with T cell chronic lymphocytic leukaemia (patient 23) stained with (a) May Grünwald Giemsa and by the alkaline phosphatase-anti-alkaline phosphatase technique for (b) T cells and (c) B cells.*

Fig. 3 *Smears of a pleural effusion from a patient with centroblastic/centrocytic lymphoma (patient 31) stained by (a) May Grünwald Giemsa and by the alkaline phosphatase-anti-alkaline phosphatase technique for (b) HLA-DR and (c) T cells. Large strongly HLA-DR positive neoplastic cells are seen, associated with much smaller reactive T lymphocytes.*

peripheral blood lymphocytes from this case). In patients 21 and 22 chronic lymphocytic leukaemia had been diagnosed on examination of peripheral blood. The pleural fluid lymphocytes appeared morphologically normal, and T cells predominated in the pleural fluid sample.

A sample from a case of T cell chronic lymphocytic leukaemia (patient 23) contained almost 100% T lymphocytes (Fig. 2), which were of T helper/inducer phenotype (in agreement with studies performed on peripheral blood lymphocytes). The cells from one case of acute T lymphoblastic leukaemia (patient 24) were also of helper/inducer phenotype.

Samples from 10 of the 12 patients with lymphoma (patients 26–33 and 35–36) contained numerous morphologically abnormal cells, and in nine patients these cells were positive for B cell markers. In most cases small normal lymphocytes reacted for the T cell marker while the abnormal larger lymphoma cells stained for B cell markers (Fig. 3). In one other sample (patient 36, histiocytic lymphoma) the tumour cells were positive with an anti-macrophage antibody, negative with the anti-B cell antibody, and showed a patchy distribution of staining with the anti-HLA-DR antibody.

Patient 25 had no cytological evidence of infiltration of the pleural cavity with lymphoma cells; the lymphoid cells looked reactive rather than neoplastic, and T and B markers did not show any abnormalities. Patient 34 contained a few scattered "immature" lymphoid cells, which appeared abnormal on routine cytological examination. Abnormal numbers of B cells were not, however, seen on immunocytochemical staining.

Discussion

Effusions containing numerous lymphocytes may be due to inflammatory processes (including tuberculosis), to non-specific reactions complicating other diseases (including carcinoma), or to chronic lymphocytic leukaemia or lymphoma.[9] In many cases it

Table 3 *Immunocytochemical labelling of serous effusions from patients with leukaemia and lymphoma*

Patient no	*Diagnosis*	*T cells*	*B cells*	*HLA-DR*	*Th*	*Ts*	*Th/Ts*	*Interpretation*	*Cytological appearances*
20	B cell chronic lymphocytic leukaemia	1	99	99	NT	NT	NT	Neoplastic B cells	Pure lymphocytic effusion
21	B cell chronic lymphocytic leukaemia	60	24	26	44	18	2·4	Reactive T cells	Carcinoma of lung; effusion contained carcinoma cells
22	B cell chronic lymphocytic leukaemia	74	21	25	64	26	2·5	Reactive T cells	Reactive effusion with mixed cell types
23	T cell chronic lymphocytic leukaemia	95	1	NT	94	1	NT	Neoplastic T cells	Pure lymphocytic effusion
24	T cell acute lymphoblastic leukaemia	99	1	1	94	1	NT	Neoplastic T cells	Abundant lymphoblasts
25	Centroblastic lymphoma	74	21	21	NT	NT	NT	Reactive T cells	No lymphoma cells identified
26	Centroblastic lymphoma	14	NT	65	NT	NT	NT	Neoplastic B cells	Mainly lymphoma cells
27	Centroblastic lymphoma	Neg*	±	±†	NT	NT	NT	Neoplastic B cells	Mainly lymphoma cells
28	Centroblastic lymphoma	Neg*	NT	Pos*	NT	NT	NT	Neoplastic B cells	Mainly lymphoma cells
29	Centroblastic lymphoma	6	NT	98	NT	NT	NT	Neoplastic B cells	Mainly lymphoma cells
30	Centroblastic lymphoma	Neg*	Pos*	Pos†	NT	NT	NT	Neoplastic B cells	Mainly lymphoma cells
31	Centroblastic/centrocytic lymphoma	Neg*	NT	Pos†	NT	NT	NT	Neoplastic B cells	Mainly lymphoma cells
32	High grade lymphoma	12	95	93	NT	NT	NT	Neoplastic B cells	Mainly lymphoma cells
33	High grade lymphoma	12	94	95	NT	NT	NT	Neoplastic B cells	Mainly lymphoma cells
34	High grade lymphoma	86	20	21	NT	NT	NT	Reactive T cells	A few lymphoma cells identified
35	Burkitt type lymphoma	1	99	99	NT	NT	NT	Neoplastic B cells	Mainly lymphoma cells
36	Histiocytic lymphoma‡	Neg*	Neg*	±†	NT	NT	NT	Probably neoplastic histiocytes	Numerous free malignant cells ?monocytic or histiocytic origin

All values represent percentages of positive lymphoid cells.
*In these cases it was not possible to make accurate counts of the percentage of positive cells—for example, because of cell clumping or thick smears. Pos and Neg refer to the reactivity of unequivocal neoplastic cells.
†±indicates patchy distribution of staining with occasional tumour cells positive.
‡Neoplastic cells from this patient were positive with EB11 (anti-macrophage antibody).
Th = helper/inducer T cells, Ts = suppressor/cytotoxic T cells.
NT = not tested.

is difficult to make a differential diagnosis on morphological criteria alone, and in these cases a study of T and B cell numbers may be important.

Although this study was based on a relatively small number of effusions, it shows clearly that immunoalkaline phosphatase labelling of T and B cell antigens in air dried smears may aid in distinguishing between reactive and neoplastic lymphoid infiltrates. The method used was applied directly to cell smears, thus avoiding the necessity to separate mononuclear cells first. This technique is essentially identical to a procedure previously described by workers in this laboratory for labelling lymphoid cell populations in blood smears[10] and has the advantage over immunofluorescence techniques that it allows simultaneous study of cell morphology and immunocytochemistry and that stained smears can be kept as a permanent record.

The results obtained in this study using cell smears may be compared to those obtained previously using separated cells and immunofluorescence. Domagala *et al*[4] reported a mean of 80·2% rosette forming lymphocytes (T cells) and 7·4% immunoglobulin bearing cells (B cells) in a control group of patients with non-malignant effusions, and Krajewski *et al*[5] detected 84% T cells and 4·5% B cells in a similar group of patients. Pettersson[11] detected T cells histochemically in pleural fluids by staining for acid α-naphthyl acetate esterase and obtained similar results.

The results of the present study are in keeping with these previous investigations in that immunoalkaline phosphatase staining with monoclonal anti-T and anti-B cell antibodies consistently showed a preponderance of T lymphocytes. It is of interest, however, that the number of HLA-DR positive lymphocytes was often slightly higher than that of B cells. This is in contrast to the results obtained in normal blood (in which B cell and HLA-DR values are essentially identical)[10] and suggests that a minority of T lymphocytes in serous effusions have undergone "activation."

The ratio of helper/inducer to suppressor/cytotoxic cells among T cells in reactive effusions (mean 3·5) was higher than that found in peripheral blood.[10] This value is closer to that seen in lymphoid tissues[12] and may reflect the migration into effusions of lymphoid cells from tissues lining serous cavities. Our results are in keeping with the single case of sarcoidosis reported by Groman *et al*,[13] in which pleural effusion cells showed a helper to suppressor ratio of 4·2. Our results conflict with those of Ginns *et al*,[14] however, who reported helper to suppressor values in pleural effusions much closer to those seen in peripheral blood.

In most cases of chronic lymphocytic leukaemia and lymphoma the patterns obtained on immunocytochemical staining were clearly different from those seen in reactive effusions. This is of potential value in the diagnosis of problem cases. The fact that staining may be performed on air dried cell smears, even after storage for long periods, means that this technique lends itself to use in the routine laboratory.

This study was supported by the Cancer Research Campaign. We are grateful to Dr PCL Beverley and Dr G Riethmuller for kindly supplying antibodies UCHT1 and T3–10 respectively.

References

1 Melamed MR. The cytological presentation of malignant lymphomas and related diseases in effusions. *Cancer* 1963;**16**:413–31.

2 Boccato P, Saran B, Pasini L, Briani G, Pasini P. Immunology of lymphocytes in pleurisy and in effusions due to pleural infiltration by chronic lymphocytic leukaemia cells. *Acta Cytol* 1978;**22**:284–5.

3 Petterson T, Klockars M, Hellström PE, Riska H, Wangel A. T and B lymphocytes in pleural effusions. *Chest* 1978;**73**:49–51.

4 Domagala W, Emeson EE, Koss LG. T and B lymphocyte enumeration in the diagnosis of lymphocyte-rich pleural fluids. *Acta Cytol* 1981;**25**:108–10.

5 Krajewski AS, Dewar AE, Ramage EF. T and B lymphocyte markers in effusions of patients with non-Hodgkin's lymphoma. *J Clin Pathol* 1982;**35**:1216–9.

6 Moir DJ, Ghosh AK, Abdulaziz Z, Knight PM, Mason DY. Immunoenzymatic staining of haematological samples with monoclonal antibodies. *Br J Haematol* 1983;**55**:395–410.

7 Cordell JL, Falini B, Erber WN, *et al*. Immunoenzymatic labelling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes). *J Histochem Cytochem* 1984;**32**:219–99.

8 Ghosh AK, Spriggs AI, Taylor-Papadimitriou J, Mason DY. Immunocytochemical staining of cells in pleural and peritoneal effusions with a panel of monoclonal antibodies. *J Clin Pathol* 1983;**36**:1154–64.

9 Spriggs AI, Vanhegan RI. Cytological diagnosis of lymphoma in serous effusions. *J Clin Pathol* 1981;**34**:1311–25.

10 Erber WN, Pinching AJ, Mason DY. Immunocytochemical detection of T and B cell populations in routine blood smears. *Lancet* 1984;i:1042–6.

11 Pettersson T. Acid alpha-naphthyl acetate esterase staining of lymphocytes in pleural effusions. *Acta Cytol* 1982;**26**:109–14.

12 Ralfkiaer E, Plesner T, Lange Wantzin G, Thomsen K, Nissen NI, Hou-Jensen K. Immunohistological identification of lymphocyte subsets and accessory cells in human hyperplastic lymph nodes. *Scand J Haematol* 1984;**32**:536–43.

13 Groman GS, Castele RJ, Altose MD, *et al*. Lymphocyte subpopulations in sarcoid pleural effusion. *Ann Int Med* 1984;**100**:75–6.

14 Ginns LC, Miller LG, Goldenheim PD, Goldstein G, Bria WF. Alterations in immunoregulatory cells in lung cancer and smoking. *J Clin Immunol* 1982;**2**:90S–4S.

Requests for reprints to: Dr AK Ghosh, Immunology Laboratory, Paterson Laboratories, Christie Hospital and Holt Radium Institute, Wilmslow Road, Manchester M20 9BX.