Papers

Monoclonal antibody JC1: New reagent for studying cell proliferation

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Abstract

Aim: To characterise a newly developed mouse monoclonal antibody JC1 which recognises a nuclear antigen present in proliferating cells in normal tissues and neoplastic lesions, and which is absent in resting cells.

Methods: The methodology was established using a representative range of frozen sections from normal tissues and from certain tumours which were immunostained with antibodies Ki67 and JC1. The molecular weight of the antigen recognised by JC1 was obtained by western blot analysis and this was compared with that of Ki67. IM-9 cell lysates containing Ki67 derived plasmids were also tested with JC1 antibody.

Results: Biochemical investigation indicated that the antigen recognised by JC1 gives two molecular weight bands of 212 and 123 kilodaltons, which is distinct from the well characterised anti-proliferation monoclonal antibody Ki67 (395–345 kilodaltons). In addition recombinant Ki67 protein is not recognised by JC1. Immunohistological reactivity was seen in areas known to contain numerous proliferating cells such as lymphoid germinal centres, splenic white matter, cortical thymocytes and undifferentiated spermatogonia. In tumours many cells from adenocarcinomas, oat cell carcinomas, squamous cell carcinomas of lung, and seminomas were labelled by JC1 with a distribution and proportion similar to that seen with Ki67. In normal tissues the only apparent difference was in testis where JC1 stained a considerably greater number of cells than Ki67. In all cases studied the new antibody showed nuclear reactivity only. JC1 did not show any cytoplasmic crossreactivity with squamous cells as is frequently seen with Ki67.

Conclusion: Antibody JC1, which recognises a nuclear antigen present in proliferating cells, should provide a useful adjunct to Ki67 as a marker of proliferation especially in those cases such as squamous cell carcinomas where a Ki67 index cannot be determined.

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The rate at which a tumour proliferates has long been considered to indicate its clinical course.[1–4] Histopathologists have therefore sought means of determining this as an adjunct to diagnosis. The development of monoclonal antibodies[5–10] recognising antigens present in cells during proliferation has brought this possibility within the realm of a general diagnostic laboratory.

The monoclonal antibody Ki67 is perhaps the best known of such reagents and has been widely used to detect proliferating cells.[10] There are now several studies which indicate a correlation between tumour proliferation rate as assessed by Ki67 immunoreactivity and clinical outcome.[11–14] However, Ki67 has a well reported crossreaction with some epithelial cells, particularly of squamous type. This can lead to strong cytoplasmic reactivity making the determination of proliferation related staining impossible to define. This has led to the elimination of cases from experimental study, and, more importantly, will not allow some individual cases to be assessed in diagnostic practice.[15–16]

Here we report the production of a new mouse monoclonal antibody (designated JC1) that is similar to Ki67 in that it seems to recognise selectively nuclei in proliferating cells. However, it differs from the latter antibody in showing little or no cytoplasmic labelling in squamous or other cell types. In addition, it does not react with the same antigen as Ki67, thus providing an independent assessment of tumour proliferation which might be valuable in clinical practice when individual cases are being evaluated.

Methods

A representative range of normal tissues was available from the frozen tissue bank stored at −70°C in the Nuffield Department of Pathology, John Radcliffe Hospital. Samples of lung cancer (10 squamous cell, seven adenocarcinomas, and three oat cell carcinomas) and seminoma (10 cases) were obtained from the same source.

Immunostaining with both monoclonal antibodies Ki67 and JC1 was performed using a three stage immunoperoxidase technique as other studies had shown that, unlike Ki67, JC1 did not perform satisfactorily with the APAAP immunoalkaline phosphatase method. The percentage of tumour cells positive for Ki67 and JC1 was established by counting 500 tumour cells from separate sections in each case.

PREPARATION OF RECOMBINANT PARTS OF THE KI67 ANTIGEN

Construction of plasmids: Nucleotides 1–1002 of the Ki67 cDNA[17] were cloned between the Eco R1 and Pst 1 sites of the pAX4a+ vector[18] (Medac, Hamburg, Germany). The resulting plasmid was designated pAX X2/1 and was transformed into E coli JM 109.[19] A 100 base pair fragment, including a 62 base pair repetitive element[17] was ligated between the Eco R1 and Bam H1 sites of the pEV-vrf vectors[20] in all reading frames. The resulting plasmids were designated pEV-vrf 1/B3, pEV-vrf 2/B3, pEV-vrf 3/B3 and transformed into E coli RR1.[21]

Preparation of bacterial cell lysates: An overnight culture of E coli JM109 containing pAX X2/1 was diluted to an OD_{550} of 0·3, grown to an OD_{550} of 1·0, and induced with 1 mM isopropylthio-β-galactoside (IPTG), according to Markmeyer et al.[18] After an additional incubation for two and a half hours at 30°C, bacteria were harvested by centrifugation, washed once in phosphate buffered saline (PBS), resuspended in 1 in 20 volume of PBS, and stored at −20°C until further use. E coli RR1 harbouring pEV-vrf 1–3/B3 plasmids were cultured at 37°C in M9 medium[22] supplemented with 40 µg/ml ampicillin, 0·5% glucose, and casamino acids, respectively, until they reached an OD_{550} of 0·8. Bacteria were then harvested and lysates were prepared for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blots, as described below.

IM-9 (multiple myeloma cell line, ATCC No CCL 159) was cultured under standard conditions in RPMI 1640 medium (Gibco, Berlin, Germany), supplemented with antibiotics and 10% fetal calf serum.

PREPARATION OF IM-9 CELL LYSATES

IM-9 cell line cells (3×10^7) were harvested by centrifugation (10 minutes at $400 \times g$) and suspended in 250 µl PBS containing 1 mM phenylmethylsulfonyl-fluoride (PMSF). The cell suspension was snap frozen with liquid nitrogen and homogenised in a mortar cooled in liquid nitrogen. The resulting powder was suspended in 300 µl double concentrated Laemmli-SDS-PAGE sample buffer[23] containing 100 mM DTT, boiled for five minutes in a water bath, and briefly sonified to destroy contaminating DNA. Samples were then subjected to SDS-PAGE.

SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli.[23] IM-9 cell lysates were run on 5% separation gels, with 3% stacking gels. Electrophoresis of the two fusion proteins was performed on 7·5 and 12·5% separation gels, respectively. Molecular weight markers were obtained from Pharmacia/LKB (Freiburg, Germany) and Sigma (Munich, Germany).

IMMUNOBLOTTING

Proteins were transferred to nitrocellulose membranes (BA 85, Schleicher & Schuell,

Dassel, Germany) using the western blot technique.[24] IM-9 cell lysates were blotted overnight at 4°C and 50 mA in a BIO-RAD blotting device (BIO-RAD, Munich, Germany); the fusion proteins were transferred in a Biometra (Gottingen, Germany) semi-dry blotting apparatus (two hours at 150 mA). Unoccupied protein binding sites of the nitrocellulose were blocked by incubation for one hour with 1% bovine serum albumin or gelatine in TRIS-buffered saline (TBS). After one hour of incubation with primary antibody and thorough washing with TBS, the bound antibody was targeted with goat anti-mouse alkaline phosphatase conjugate, diluted 1 in 10 000 in blocking solution. The phosphatase activity was visualised using nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as substrates.[25]

Results

CHARACTERISATION OF THE JC1 ANTIBODY AND ANTIGEN

Biochemical characterisation of antibody JC1

Western blot analysis of IM-9 cell lysates showed that there were two bands with molecular weights of 212 and 123 kilodaltons (fig 1A). These are quite clearly different from the bands seen with Ki67 on the same cell lysate preparation (fig 1A). Additional evidence that JC1 antigen is distinct from Ki67 antigen is shown by western blotting with both antibodies of protein preparations prepared from the transfection of Ki67 c DNA plasmids into E coli. In no case were any of the resultant fusion proteins recognised by JC1 (figs 1B and C).

Normal human tissues

In human tonsils (figs 2A and B) antibody JC1 stained the nuclei of nearly all centroblasts in the dark zone, a varying number of centrocytes in the light zone of germinal centres, and the nuclei of only a small number of follicular mantle lymphocytes. This pattern of staining was virtually identical with that seen in parallel sections labelled by antibody Ki67. In the overlying squamous epithelium of the tonsil (figs 3A and B) there was a distinct difference in the staining pattern seen with JC1 and Ki67. With Ki67 there is a strong cytoplasmic reactivity with the epithelial cells especially of the basal and intermediate layers. This makes it difficult to appreciate the nuclear labelling. However, Ki67, in general, seems to label nuclei preferentially in the basal layer. JC1 has no cytoplasmic crossreactivity so that nuclear staining is clearly seen as being localised almost exclusively in the intermediate layer, with only an occasional positive nucleus being observed in the basal layer.

In two other lymphoid organs, thymus (figs 4A and B) and spleen, both antibodies gave a similar staining pattern in the areas known to contain the most proliferating cells—that is, the cortex of the thymus and the white matter in the spleen.

In specimens of five different testes, a sharp difference was observed between Ki67 and
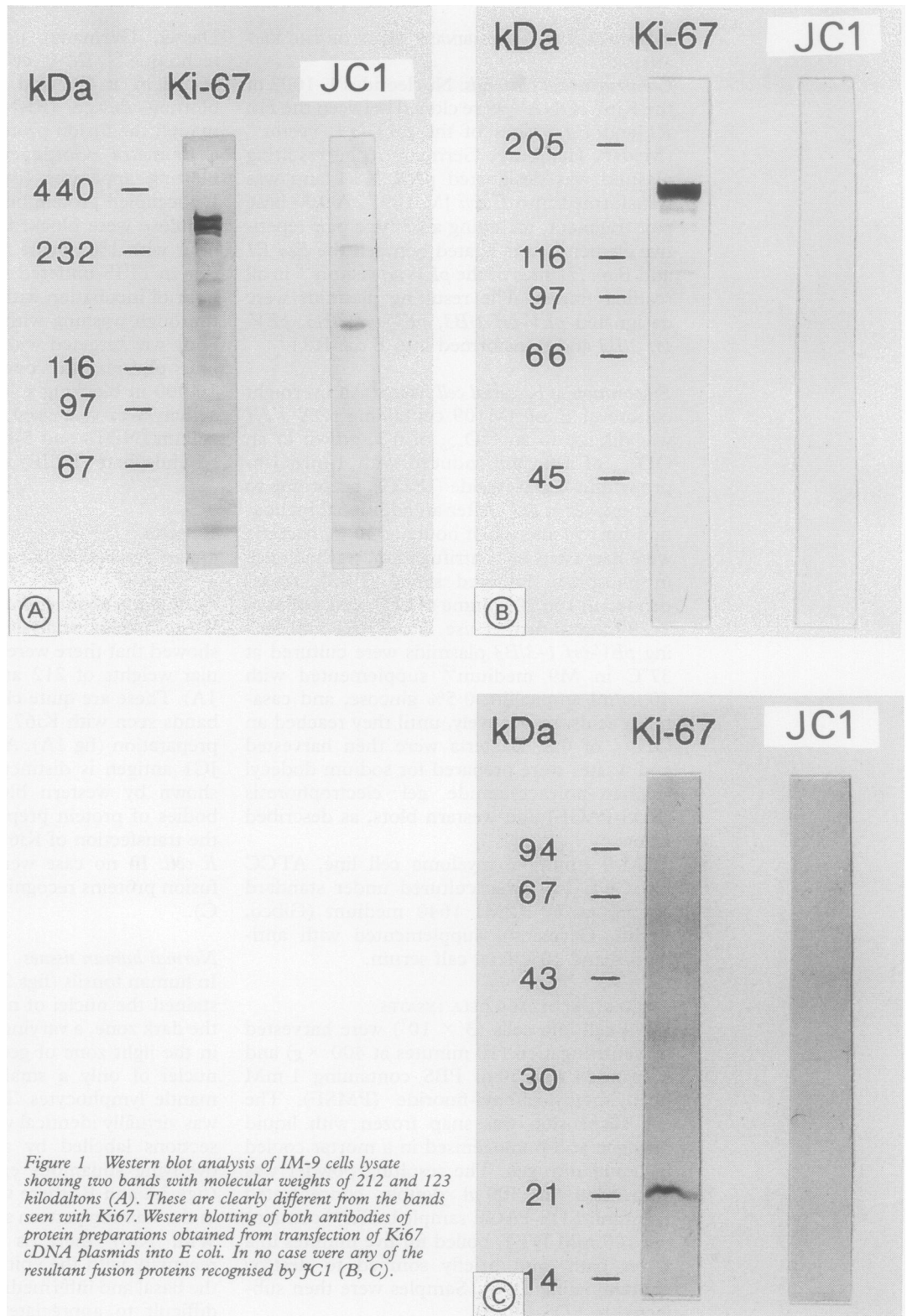
Figure 1 Western blot analysis of IM-9 cells lysate showing two bands with molecular weights of 212 and 123 kilodaltons (A). These are clearly different from the bands seen with Ki67. Western blotting of both antibodies of protein preparations obtained from transfection of Ki67 cDNA plasmids into E coli. In no case were any of the resultant fusion proteins recognised by JC1 (B, C).

JC1. Ki67 generally stains only a small number of undifferentiated spermatogonia located in the basal layer of the seminiferous tubule (fig 5A). JC1 labels a much larger number of undifferentiated spermatogonia extending towards the lumen with additional staining of some spermatocytes S1 and spermatides S3 (fig 5B). Neither of the two antibodies stained mature spermatozoa or Leydig cells.

In other normal tissue, comprising liver, kidney, pancreas, thyroid, prostate, lung and brain, only an occasional nucleus was labelled by either antibody and no difference was seen in their distribution or number. In proliferating

normal tissues like gut or bone marrow, no difference in quantity or quality of staining between the two antibodies was noted. Table 1 summarises the results.

Malignant lung and testicular tumours
The number of labelled nuclei per 500 cells counted for each tumour type is shown in table 2. There was no significant difference between the two antibodies in the adenocarcinomas and oat cell carcinomas of the lung (figs 7A and B). In five of the cases of squamous cell carcinomas of the lung there was also no difference in staining between Ki67 and JC1. However, in
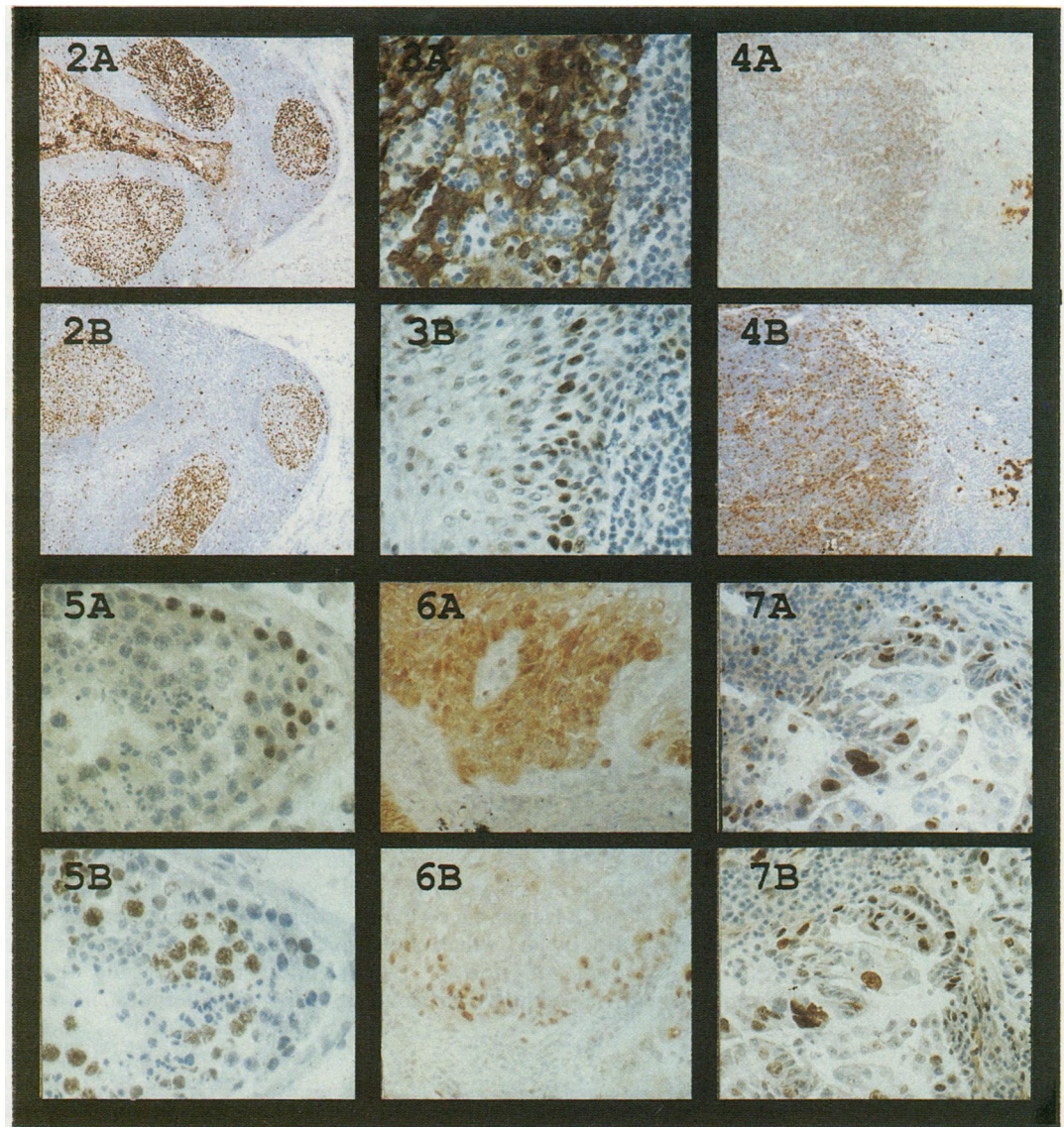
Figures 2A, B Immunostaining of human tonsil with both Ki67 (A) and JC1 (B), respectively. Lymphoid follicules show virtually identical pattern of staining by both antibodies in parallel sections, with strong staining of the germinal centres and a few positive cells from the mantle zone. (Three stage immunoperoxidase technique).

Figures 3A, B Reactivity of the overlying squamous epithelium of tonsil: Note the difference in the staining pattern between Ki67 (A) and JC1 (B). Ki67 showing a strong cytoplasmic cross reactivity of the epithelial cells from the basal and intermediate layers making the nuclear labelling difficult to appreciate. Occasional nuclei from the basal layer are stained by Ki67. In contrast, JC1 shows no cytoplasmic cross-reactivity but clear nuclear staining is localised in the intermediate layer with only an occasional nucleus being observed in the basal layer. (Three stage immunoperoxidase technique).

Figures 4A, B Immunostaining of the human thymus: both antibodies, Ki67 (A) and JC1 (B) show strong positive labelling of cortical thymocytes. (Three stage immunoperoxidase technique).

Figures 5A, B Immunostaining of human testis: Ki67 (A) shows labelling of a small number of undifferentiated spermatogonia from the basal layer of the seminiferous tubule contrasting with JC1 (B) which shows labelling of a larger number of undifferentiated spermatogonia extending towards the lumen of the seminferous tubule. (Three stage immunoperoxidase technique).

Figures 6A, B Reactivity of squamous cell carcinoma of the lung: Ki67 shows pronounced background staining (A) which obscures the nuclear labelling. A parallel section from the same case stained with JC1 (B) shows reactivity confined to the nuclei. (Three stage immunoperoxidase technique).

Figures 7A, B Immunostaining of a typical case of adenocarcinoma of the lung: both antibodies show almost identical patterns of staining with Ki67 (A) and JC1 (B).

the remaining five cases it was only possible to achieve a count of nuclei with JC1 because the level of background staining with Ki67 was so high. These cases had been selected from previous studies of Ki67 labelling in lung cancer where they had been eliminated from inclusion due to this effect (figs 6A and B).

In view of the difference noted betwen Ki67 and JC1 on normal testis 10 cases of seminoma were selected for study. There was a consistent difference, with a higher number of cells being labelled by JC1 (table 2). However, this difference was not significant ($p > 0.134$) and there was no evidence of the striking difference in distribution of the staining by the two antibodies as was seen in normal testis.

Discussion

Several monoclonal antibodies that identify a variety of cell cycle related antigens have recently been described as markers of pro-

Table 1   Staining characteristics of Ki67 compared with JC1 in sections from normal human tissues

	Ki67	JC1
Squamous epithelium overlying tonsils		
Basal layer	+ + + +*	+/-
Intermediate	+*	+ + +
Granular layer	-	+ +
Tonsils:		
Germinal centres	+ + + +	+ + +
Testis:		
Undifferentiated spermatogonia	+ +	+ + +
Differentiated	-	+ +
Skin:		
Basal layer	+ + + +*	+/-
Intermediate	+*	+ + +
Granular layer	-	+ +
Kidney	-	-
Liver	-	-
Brain	-	-
Lung	-	-
Thyroid	-	-
Pancreas	-	-
Prostate	-	-
Bone marrow and gut	All proliferative compartments	

Key: + + + >80% of cells show nuclear staining.
\+ + +   50-80% of cells show nuclear staining.
\+ +   10-50% of cells show nuclear staining.
\+   <10% of cells show nuclear staining.
+/-   Only occasional cells show nuclear staining.
-   Negative staining.
*Cytoplasmic staining also seen.

liferation—Ki67,[26] anti-PCNA.[9][27] Of these, Ki67 has been well characterised as recognising polypeptide chains with molecular weights of 395 and 345 kilodaltons present in the nucleus of cells in all stages of the cell cycle except G_o.[17][28] We have produced a new mouse monoclonal antibody JC1 which shows nuclear reactivity in cells of normal tissues and tumours, with a general distribution very

Table 2   Staining characteristics of malignant tumours with Ki67 and JC1

Case No	Ki67 (%)	JC1 (%)
Adenocarcinoma of the lung:		
1	12	6
2	26	27
3	8	9
4	31	36
5	20	24
6	31	54
7	No stain	10
(p > 0·771)		
Oat cell carcinoma of lung:		
8	35	40
9	35	30
10	46	36
Squamous cell carcinoma of lung:		
11	32	34
12	34	27
13	36	23
14	52	38
15	41	41
16	-*	28
17	-*	23
18	-*	23
19	-*	17
20	-*	32
(p > 0·228)		
Seminomas:		
21	34	62
22	25	21
23	30	72
24	13	18
25	22	15
26	34	41
27	16	23
28	31	56
29	42	53
30	48	49
(p > 0·134)		

*Due to high background cytoplasmic staining, a nuclear count for Ki67 was not possible in cases 16-20.

similar to that seen with Ki67,[26] for example in tonsil and thymus, bone marrow and gut. However, we know from the molecular weight of the JC1 antigen that it differs from Ki67. This is reflected in two important observations made in the current study. The first is that JC1 does not cross-react with a cytoplasmic antigen in squamous epithelium. The second is that the distribution of nuclear staining is not identical with Ki67 in all normal tissues such as testis and skin.

The lack of cytoplasmic staining with JC1 on squamous epithelium may be of considerable value in studies of squamous tumours. Several studies have reported this effect with Ki67 which has led to the elimination of some cases from evaluation; examples include cervical cancers[16][29] and lung carcinomas.[15] Furthermore, this effect has also been noted in breast carcinoma[30] and haematological diseases.[31] Although these were not specifically addressed in the present preliminary survey with JC1, there is no reason to suppose that it would occur with this antibody. In this study we specifically selected a number of squamous cell carcinomas of the lung that we had previously been forced to eliminate from our investigations with Ki67. In each case JC1 gave a clear nuclear staining without any cross-reaction. Because JC1 correlated well with Ki67 in the other squamous cell carcinomas and in the adenocarcinomas and oat cell carcinomas, it is clear that a combination of these two antibodies should be valuable in future studies of proliferation in these tumour types. Furthermore, if it is shown that a measurement of tumour growth fraction is useful in determining some aspect of tumour behaviour, such as prognosis or drug sensitivity, it will be important to have an antibody such as JC1 for study of those cases which cannot be evaluated with Ki67 alone.

The second major difference from Ki67 concerned mainly the testis. JC1 stained a larger and different number of undifferentiated spermatogonia than Ki67. The reason for this is unknown but must obviously reflect a distinction in the localisation of the two antigens in testis which is not detectable in most other tissues. Interestingly, this did not seem to affect the measurement of proliferating cells in the series of seminomas, a malignancy directly arising from seminiferous tubules. However, it emphasises that caution must always be observed in comparing results from antibodies recognising different antigens and that future studies would do well to incorporate both antibodies or at least another independent measure of proliferation.

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