The Carapace of the Cancer Cell

The Osler Oration 1984

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Introduction

In the long line of Regius Professors of Medicine at Oxford, I think no pair could be as dissimilar as Osler and myself. Apart from the immense disparity of personal style, Osler was, above all else, a clinician, whereas I am an experimentalist. Indeed, it is well known among my colleagues at Oxford that my clinical skills are more meagre than had hitherto been thought possible. There is thus no chance that Osler could be one of my heroes. But, looking at him more closely since a misguided hand moved me into the chair that he held with such lustre, I found, to my surprise, that I shared many of his views on what was important in determining medical progress, and I also found, to my even greater surprise, that some of my heroes were also his. He said[1] that highly trained observation alone cannot bring us much further than the stage reached by the ancient Greeks, and that the revolutionary progress of modern medicine in alleviating pain and eliminating disease is due essentially to the application of the experimental method. Among the names that he chose for special admiration are Copernicus, Kepler, Galileo, Jenner, Pasteur and Koch. Although a supreme practitioner of the Art of medicine, Osler had no doubt that the Art was incomparably less important than the Science. In lecturing on my experimental work, I like to think that Osler would not have disapproved.

Hybrid Cells as a Screen for Markers of Malignancy

The work discussed is based on the technique of cell fusion developed by John Watkins and myself almost two decades ago[2]. When somatic cells are fused together, they generate composite or hybrid cells which have found very wide application in biology and medicine: in the mapping of genes, in the investigation of hereditary human diseases, in the analysis of genetic regulation and differentiation, and, more recently, in the study of antibody formation, the last having yielded, as a by-product, a routine method for the production of monoclonal antibodies of pre-determined specificity[3]. These hybrid cells have also been used to explore, at the cellular level, the genetic determinants of malignancy, defined, in this context, as the ability of a cell to multiply progressively in a compatible host and kill it. It has been found that when any one of a wide variety of malignant cells, either rodent or human, is fused with a normal diploid fibroblast of the same species, the resulting hybrid cell, so long as it retains the chromosome complements of the two parent cells, is unable to grow progressively and kill a compatible host: the malignant phenotype is suppressed[4-6]. Although a few contrary observations have been reported, a recent review[7] concludes that there is now no well-authenticated exception to this rule. However, on continued cultivation in vitro, all hybrid cells eliminate chromosomes at varying rates; and when certain specific chromosomes donated by the diploid fibroblast (the suppressor chromosomes) are eliminated from the hybrid, the malignant phenotype reappears[8,9]. In this way, isogenic pairs of hybrid cells, in which one member of the pair is malignant and the other is not, can be generated. Such matched pairs of hybrid cells can be used as screens for markers associated with the malignant phenotype. One would be interested in any marker present in the malignant parent cell (but not in the normal diploid fibroblast) which disappears when malignancy is suppressed in the hybrid and reappears when malignancy returns as a consequence of chromosome loss. The hybrid cell screen is thus essentially a genetic segregation test. Using the hybrid cells in this way, we have detected a number of markers that are closely associated with malignancy, of which the most interesting, from a functional point of view, is a characteristic change that tumour cells show in the kinetics of glucose uptake[10]. However, I shall here describe another marker that was discovered by the application of immunological techniques to the hybrid cell screen.

Ca1: a Monoclonal Antibody detected in the Screen

The hunt began with the partial purification from extracts of a human laryngeal carcinoma cell line (Hep2) of membrane proteins or groups of membrane proteins in which, as a consequence of previous work, we had a special interest. (We, in this context, were R. L. Gingrich, F. Ashall, M. E. Bramwell, V. P. Bhavanandan, G. Wiseman and myself.) Using these partially purified preparations as immunogens, we generated a range of monoclonal antibodies and tested them against a panel of matched pairs of human hybrid cells of the kind I have described. It is not difficult to find monoclonal antibodies that show quantitative differences in the extent to which they bind to the malignant, as opposed to the non-malignant, partners in the hybrid cell screen; but anti-
bodies that discriminate absolutely between such partners are very rare. Indeed, only two have so far been discovered. One of these is directed against a membrane phosphoprotein with an estimated molecular mass of 75,000[11]; the second is the antibody we call Ca1 because it was the first that we had found to distinguish between malignant and non-malignant cells in our matched hybrid cell screen[12].

The Ca Antigen

Ca1 is an IgM antibody that precipitates from cell extracts two components which separate in electrophoresis in positions corresponding to molecular masses of approximately 390,000 and 350,000. We call these two components the Ca antigen. Preparations from different cell lines show some heterogeneity on electrophoresis, a phenomenon that we initially regarded as trivial but that subsequently proved to be of interest. These antigenic components were found to have the characteristics of glycoproteins of high molecular weight. Their antigenicity was unaffected by boiling or extraction by the common organic solvents, but it was destroyed by thorough desialylation with neuraminidase or by prolonged digestion with the proteolytic preparation, Pronase. Although the immunogen was isolated from a single human malignant cell line, the Ca antigen was found to be present in all of about a dozen cell lines derived from human cancers; and the Ca1 antibody reacted in immunohistological tests with a very wide range of malignant human tumours examined in sections of clinical material[13]. Because of the stability of the antigen to heat, and to extraction by organic solvents, the antibody was found to be applicable to routine paraffin sections, which greatly facilitated the screening of pathological specimens. In the normal tissues of the human body, staining by Ca1 was found at the following sites:
1. the transitional epithelium of the urinary tract and, to some extent, the collecting tubules of the kidney;
2. the luminal epithelium of the Fallopian tube;
3. the epithelium of apocrine sweat glands and the ducts of eccrine sweat glands;
4. the trophoblast of the developing embryo; and
5. type 2 pneumonocytes.

We shall see later whether it might not be possible to discern a connecting strand in this apparently haphazard pattern of physiological staining; but it is helpful to consider first what we have found out about the structure of the Ca antigen.

Structure of the Antigen

Malignant cells growing in vitro shed the antigen into the medium, so that cells and medium may serve as starting material for purification procedures. The structural studies presented here were done on preparations of the antigen isolated from deoxycholate extracts of the human laryngeal carcinoma cell line by affinity chromatography on columns of the Ca1 antibody coupled to Sepharose 4B beads. The antigen eluted from the affinity columns was further purified by high performance liquid chromatography, gel filtration and chromatography on wheat germ agglutinin-Sepharose columns (the carbohydrate moiety of the Ca antigen binds to wheat germ agglutinin). The elution pattern of the purified antigen on a variety of chromatography columns confirmed its high molecular weight and suggested further that it contained a very high proportion of carbohydrate. This was supported by measurements of the dry mass of the purified antigen before and after deglycosylation. These measurements indicated that the antigen consisted of about two-thirds polysaccharide and one-third polypeptide. Polycyacylamine gel electrophoresis of the deglycosylated antigen gave, in our best (least degraded) preparation, a molecular mass of about 100,000 for the polypeptide moiety. The behaviour of the purified antigen on ion-exchange columns excluded the possibility that it was either sulphated or phosphorylated. The amino acid composition of the purified polypeptide showed very high proportions of serine, glycine and glutamic acid.

Structural analysis of the polysaccharide, which was done by V. P. Bhavanandan, showed that the antigen had the characteristic features of a mucus-type glycoprotein or mucin: at least 95 per cent of the oligosaccharides are 0-glycosidically linked to the polypeptide. The major sugars present were N-acetylenuraminic acid (NeuNac), N-acetylglactosamine (GalNac) and galactose (Gal); and virtually all of the polysaccharide could be accounted for by oligosaccharides having the structure (NeuNac)n→(Gal)m→(GalNac), where n = 0, 1 or 2; that is, a simple disaccharide (Gal→GalNac) to which either 2, 1 or no sialic acid residues were attached. As far as I am aware, this is the simplest mucin that has yet been isolated from a human source; indeed, of all mucins so far characterised, only ovine submaxillary mucin is simpler. (For further details of the structural analysis, see [14]).

The Ca Antigen in Normal Tissues

In the Ca antigen we are therefore dealing with a mucin that is found in a very wide range of malignant tumours, but in only a few, very specialised, sites in the normal body, for the most part epithelia in direct contact with fluids or secretions having physico-chemical characteristics very different from those of the normal extracellular fluid. We began our studies of the possible function of the Ca antigen by examining in some detail the component stained by the Ca1 antibody in sections of transitional epithelium of the bladder. This was the first site in normal tissue at which a specific reaction with the Ca1 antibody was found. It was obviously of cardinal importance to determine whether the component reacting with the Ca1 antibody in normal tissues was the same as the antigen isolated from malignant cells, or whether cross-reactions were occurring. For a simple technical reason, the transitional epithelium of the bladder appeared, at first glance, to be a more promising starting material than most other normal tissues. Because this epithelium consists of several layers of cells, it is possible to strip it away from the bladder and use extracts of the stripped epithelium, largely free from contamination with other cell types, as a primary source of antigen. To avoid problems that might
arise as a consequence of autolysis, we used specimens of bladder and ureter removed at operation and frozen at once in liquid nitrogen. Purification of the antigenic material by affinity chromatography on columns of the Ca1 antibody, and examination of the purified material by gel electrophoresis and other methods, revealed that it was indeed the Ca antigen: like the material extracted from tumour cells, it separated in electrophoresis as two components with approximate molecular masses of 390,000 and 350,000.

We examined the distribution of the antigen in the urothelium by immunohistological procedures applied to sections of rapidly fixed material removed at operation. The antigen is not seen in the basal generative layers of the epithelium. Its synthesis is initiated in the intermediate (pyriform) layer, where it appears to accumulate preferentially on the luminal aspects of the cells; and it is present in highest concentration in the cells that constitute the luminal surface. Here the mucin forms a dense continuous layer. In the epithelium of the sweat glands and the Fallopian tube, the Ca antigen is also distributed as a continuous lining on the luminal surface. It seemed probable from its location in the urothelium that the antigen would be shed into the urine. A sample of urine was therefore concentrated, and the concentrate extracted in the usual way by affinity chromatography on a Ca1 antibody column. The Ca antigen, showing the characteristic bands on gel electrophoresis, was indeed found to be present.

Polymorphism of the Antigen

I mentioned before that there was some heterogeneity in the electrophoretic pattern of the Ca antigen in polyacrylamide gels, which we initially ascribed to the microheterogeneity commonly observed in glycoproteins with a high carbohydrate content. However, in March 1983 I received a note from Professor Walter Morgan, then in his eighty-second year, who had listened to a talk I had given on this antigen. He drew my attention to a urinary mucin which had just been described by Cartron and his colleagues[15], and which seemed to him to have similar properties to our antigen. A little later, Dr Dallas Swallow, of the MRC Human Biochemical Genetics Unit at University College, asked whether I thought it possible that the Ca antigen we had found in the urine might not be the peanut lectin-reactive urinary mucin (PUM) which she and her colleagues had been studying in collaboration with Cartron and which they had found to show a systematic genetic polymorphism in human populations[16]. We collected a range of urine samples from different individuals and examined the Ca antigen purified from each of them. This survey showed that the Ca antigen, as detected by our Ca1 antibody, did indeed show the same electrophoretic polymorphism as Swallow and her colleagues had described. There now seems little doubt that the Ca antigen in the urine and the peanut lectin-reactive urinary mucin are one and the same thing. We have not yet purified the Ca antigen from other normal epithelia, but we have extracted it from sweat, where it has the same electrophoretic properties as the material isolated from urine, and where it also shows the characteristic genetic polymorphism. It therefore seems very probable that the few normal epithelia stained by the Ca1 antibody in immunohistological procedures do indeed express the Ca antigen on their luminal surfaces.

Function of the Antigen

These observations, coupled with the information I have presented about its structure, lead me to suppose that the Ca antigen serves a function that is classical for a mucin, namely to shield the epithelium producing it from toxic agents that might otherwise be destructive. What, in the case of urine, for example, might these toxic agents be? Since, normally, the urine secreted by the kidney is essentially a protein- and cell-free filtrate, we do not have to consider a cellular or enzymatic attack on the urothelium under physiological conditions. Since the literature provides little information about the cytotoxicity for epithelial cells of the non-volatile solutes of the urine, we examined this question directly by adding increasing concentrations of urine to epithelial cells growing in vitro. Even at a concentration of 15–20 per cent of urine in the medium, the growth of these cells was hardly impaired. It thus seemed very unlikely that the non-volatile solutes of urine had any significant acute toxicity for epithelial cells. However, the pH of excreted urine usually lies within the range of 4.5 to 6.0; and the pH of sweat may also fall to well below 5.0[17]. Since hydrogen ion concentrations below pH 5.0 are rapidly lethal to almost all mammalian cells in vitro, it seemed possible that at least one function of the Ca antigen might be to shield epithelial cells from extremes of pH. I have not been able to obtain systematic information about the pH of the contents of the human oviduct under physiological conditions, but low pH values have been reported in animals[18]; and it is not improbable that low extracellular pH may also be found in the vicinity of the trophoblast and in pulmonary conditions associated with the activity of type 2 pneumocytes. It seemed unlikely, however, that production of the Ca antigen would actually be induced by the fall in pH; for, in the urothelium, synthesis of the antigen is initiated in the intermediate layers which are already shielded from the low pH of the urine.

Induction of the Antigen by Lactate

In thinking this problem over, it occurred to me that lactate might play a role. High lactate concentrations are characteristic of the secretions of the sweat glands[17], and of the oviduct[19,20], where the lactate is apparently generated by the glycolytic activity of the epithelial cells themselves[21]; and there is evidence that this may also be the case for the cells of the urothelium and for type 2 pneumocytes. The effect of high lactate concentrations, at physiological pH, was therefore examined in a selection of tumour cell lines. In most, the added lactate produced only small effects, if any at all; but, in one cell line, derived from a human bladder carcinoma, the addition of lactate provoked a marked increase in the amount of Ca antigen produced by the cell. This increase
affected both intracellular antigen and antigen on the cell surface, where, in some experiments, a more than twenty-fold increase was observed. This induction of the Ca antigen by lactate is now being studied in detail. It is dose and time-dependent, probably represents de novo synthesis of the whole mucin molecule, and is gratuitous in that it does not require metabolism of the added lactate. D-lactate, the non-physiological isomer, which is not metabolised by cells growing in vitro, is as effective as the physiological isomer, L-lactate. We thus have cells that make the Ca antigen constitutively, cells in which it can be gratuitously induced by lactate, and cells, such as diploid fibroblasts, which do not make it at all, even in the presence of lactate. For those familiar with the lactose operon in E. coli, it will at once be apparent that we have here a very attractive situation for further analysis by the techniques of somatic cell genetics; and we are, of course, already deeply involved in this. Since almost nothing is known at the level of the cell about the mechanisms that control the production of mucins, it is a very gratifying by-product of our work on malignancy that we have thus been given an entirely novel approach to this hitherto intractable problem.

Significance of the Antigen in Malignant Tumours

What then is the significance of the Ca antigen in malignant tumours? In tumours derived from epithelia that normally synthesise the antigen, we may assume that its continued synthesis is simply an example of persistence of the differentiated trait in the malignant cells. But a very wide range of different malignant tumours, not derived from such epithelia, also express the antigen, either throughout the tumour or focally, in patches of cells of varying size. In these tumours, I suspect that we are dealing with ectopic production of the antigen; but I do not believe that this ectopic production is without biological significance. If we accept that the Ca antigen has a protective role in the epithelia that normally synthesise it, especially as a shield against hydrogen ion concentrations outside the tolerable physiological range, then it is reasonable to suppose that this mucin may serve a similar function in malignant cells. A recent detailed study has indicated that, associated with an impaired or otherwise inadequate blood supply, the glycolytic activity of malignant tumours may generate very high concentrations of lactate and very high extracellular concentrations of hydrogen ions[22]. It seems possible that foci within malignant tumours where the Ca antigen can be detected may well be sites at which its synthesis has been induced by the accumulation of lactate. However, most cell lines derived from malignant tumours synthesise the antigen constitutively in vitro, although in greatly varying amounts. It is therefore possible that constitutive production may also be a feature of some malignant tumours in vivo. If the Ca antigen does have the protective role we envisage, how are we to explain its absence from malignancies such as melanomas, tumours of the central nervous system, and most carcinomas of the gastrointestinal tract[13]? I suspect the answer to this question is not that these tumours dispense with a protective mechanism of this kind, but that they achieve the same end by producing other mucins which have a similar function but are not recognised by the Ca1 antibody. There is good evidence that malignant tumours are able to synthesise a substantial repertoire of mucins, and it is not to be expected that they will all be recognised by the one monoclonal antibody. There is, for example, strong chemical evidence that human melanomas synthesise a mucin that is structurally very similar to the Ca antigen[23], but the Ca1 antibody does not bind to human melanomas or to cell lines derived from them. The production of a wider range of monoclonal antibodies directed against the mucins produced by different kinds of tumour might well show that the Ca antigen is only one member of a family of related molecules that perform similar functions.

Clinical Applications

It seems appropriate to conclude with a few words about the present state of possible clinical applications of the Ca1 antibody. Due to the absence of the Ca antigen in some malignant tumours, its patchy distribution in others, and its presence in certain specialised normal epithelia, it does not now seem probable that the Ca1 antibody will make an important contribution to the diagnosis of tumours in routine sections of pathological material. It does, however, appear to be of substantial value in the identification of malignant cells in exudates and serous fluids. An extensive study by Spriggs and his colleagues[24-26] has demonstrated not only that the Ca1 antibody is useful in identifying equivocal or putatively malignant cells in cytological preparations, but also in revealing malignant cells where they were previously overlooked. In comparison with another monoclonal antibody that has been tested for this purpose, the Imperial Cancer Research Fund's HMFG-2 antibody, directed against an as yet incompletely characterised epithelial antigen, the Ca1 antibody was found to give remarkably few false positives[27]. We now have Ca2 and Ca3, IgG monoclonal antibodies produced by immunisation with purified preparations of the Ca antigen. These new antibodies react, at least in part, with different sites on the Ca antigen, and have certain properties that make them better reagents for cytological preparations than our original Ca1. These new antibodies are now undergoing clinical trial, and the initial results look most promising. Indeed, I have Dr Spriggs' permission to describe them as, in his view, the best antibodies at present available for the identification of malignant cells in cytological preparations.

Finally, there is one further characteristic of the Ca antigen that may prove to be of clinical use. I have mentioned that malignant cells growing in vitro shed the antigen into the medium. Malignant cells growing in vivo shed the antigen into the blood stream, where its presence may be sensitively detected by radioimmunoassay. The antigen is present in blood at a low level under normal conditions, but, with some malignant tumours, much higher levels are found. Unfortunately, this is also true for some non-malignant clinical conditions in which there
is proliferation of the cells that normally synthesise the antigen. Nonetheless, it seems possible that serial measurements of the Ca antigen levels in the blood before and after surgical removal of a malignant tumour may, in some cases, provide a sensitive measure of the continued presence or the subsequent development of metastatic deposits. Even the most intransigent of academic experimentalists must confess that he is not displeased if his work proves to have some clinical application.

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