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Monoclonal antibodies showing sequence specificity in their interaction with single-stranded DNAs

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ABSTRACT

Six hybridoma cell lines which secrete monoclonal antibodies binding to nucleic acids were produced from autoimmune NZB/NZW mice. Four of the antibodies were IgG's and the other two were IgM's. Using a solid phase radioimmunoassay (SPRIA) the binding of the antibodies to over thirty different nucleic acids was estimated. All the antibodies were extremely specific. There was no detectable interaction with various RNAs, and single-stranded DNAs bound more antibodies than duplex or multi-stranded DNAs. In every case the antibodies also showed considerable sequence preferences. For example one monoclonal antibody bound to $d(TTC)_n$ but not to $d(TCC)_n$ while another interacted strongly with $d(TG)_n$ and $d(CA)_n$ but not with $d(TC)_n$, $d(GA)_n$ or homopolymers. In other cases the patterns of sequence specificity were extremely difficult to interpret although it seems clear that monoclonal antibodies have the potential to distinguish between any two nucleic acids however similar.

INTRODUCTION

Antibodies to nucleic acids are of interest for several reasons. First they play a significant role in autoimmune diseases such as Systemic Lupus Erythematosus (SLE) (1). Second, they can provide a model system for protein recognition of nucleic acids in general. Finally, they can be used as probes for particular nucleic acid sequences or structures in more complex systems such as ribosomes or chromosomes (2,3). Before the introduction of the hybridoma technology, antibodies were obtained directly from immunized animals. These antibodies often show a wide range of specificities which in some cases limits their usefulness. Moreover, the degree of specificity of individual members of these heterogeneous antibody populations

is not yet clear. For example it has been shown previously (for a review see 4) that structural features of nucleic acids can be distinguished (e.g. duplex or triplex) but it has yet to be demonstrated that sequence specificity can also be achieved. To attempt to answer this question we have produced several hybridoma cell lines which secrete monoclonal antibodies which bind to nucleic acids, and have investigated their specificities in detail.

MATERIALS AND METHODS

Four 6 1/2 month old female NZB/NZW mice, (a gift of Dr. A.S. Russell, Dept. of Medicine, University of Alberta) were used for a single cell fusion. At this age the mice begin to develop an autoimmune disease resembling systemic lupus erythematosus and antibodies against various nucleic acids can be detected in the sera (5). Two mice were injected with 25µg of lipopolysaccharide (LPS) and two with 50µg and then 48 hours later the spleen cells were harvested for fusion with clone 43 of the mouse myeloma MOPC 315 (7). This cell line synthesizes but does not secrete Ig light chains (6,7). LPS treatment, in general, leads to a larger number of viable hybridomas and will be described in detail elsewhere (8). The cell fusion technique, limiting dilutions and cloning in agarose were performed essentially as described previously (6,7,9). After 14 days when a total of approximately 1,000 individual clones could be visualized, screening for the production of nucleic acid binding antibodies was initiated using a Solid Phase RadioImmunoAssay (SPRIA) described below.

Polyvinyl chloride 96-well flat-bottomed microtiter plates (Dynatech Laboratories Inc.) were coated with various nucleic acids in 50µl of PBS buffer (2.7 mM KCl, 137 mM NaCl. 10 mM Na_2HPO_4, 1.4 mM KH_2PO_4 final pH of 7.2) at 4^{o} for at least 24 hours. Using ^{3}H-labelled nucleic acids it was found that approximately 1ng of nucleic acid becomes permanently attached by some unknown mechanism. For the initial screening of the clones two groups of antigens were used.

Group A: - 1µg/ml rU_n, 1µg/ml dT_n, 1µg/ml heat-denatured ribosomal RNA from *E. coli* and 1µg/ml

heat-denatured calf thymus DNA.

Group B: - 1µg/ml R17 RNA, 1µg/ml native calf thymus DNA, 1µg/ml $rU_n \cdot dA_n \cdot dT_n$ and 1µg/ml $rA_n \cdot dT_n$. For the specificity tests the wells were coated with 50µl of 1µg/ml of the appropriate nucleic acid as above. After washing three times with 100µl of PBST (PBS buffer with 0.05% Tween) to remove unbound nucleic acid, 25µl of PBST was added followed by 20-100µl of the sample to be tested. After incubation at 20°C for 2 hours the wells were again washed three times with 100µl of PBST to remove unbound antibodies. 30-50µl of I^{125}-labelled goat anti-mouse IgG (10) was then added and incubated for a further 2 hours. Thus the I^{125} reagent bound to any monoclonal mouse antibodies which were bound to the nucleic acids on the plate. The plate was finally washed three times with 100µl of PBS to remove excess I^{125} reagent. The wells were excised with a hot-wire cutter and counted in a LKB-Wallac 1270 Backgamma II, usually for one minute.

The source of the nucleic acids used in this study have been described previously (11,12). In general single-stranded pyrimidine DNAs were prepared by depurination of the duplex, whereas single-stranded purine DNAs were prepared by exonuclease III digestion of the appropriate duplex (12,13).

Gel exclusion chromatography was performed on a 140ml column of Sephadex G-200. Approximately 10ml of the hybridoma growth medium was precipitated with 50% $(NH_4)_2SO_4$ and resuspended in 1 ml of the eluting buffer (1 M NaCl, 100 mM Tris-HCl, pH 8.0) The sample was eluted at 9 ml/hour and fractions were monitored at 280 nm as well as by the SPRIA assay described above.

RESULTS

The fused cells from the spleens of the two groups of mice were plated out into 192 individual wells to allow clones to develop. After approximately one week individual clones could be directly visualized and there were on average about five per well (2.0 ml). There was no significant difference in the number of clones originating from the two groups of mice receiving different doses of LPS. After ten days the SPRIA

assay was used to identify those wells which were producing antibodies which bound to either the A or B group antigens. 31 wells were found to be positive (defined as giving a result in the SPRIA assay which was two-fold or more above background). This was repeated two days later using one third the volume of medium from each well for the SPRIA assay. On this occasion only 21 of the original 31 wells were still significantly positive. In both cases all wells which were found positive for the B group antigens were also positive for the A group. Moreover the interaction with the A group antigens as judged from the CPM in the SPRIA assay were always significantly greater than for the B group. This immediately suggests, on comparing the two groups of antigens, that the majority of the hybridomas were secreting antibodies specific for single-stranded DNAs. Finally after using limiting dilutions and cloning in agarose (9) to ensure monoclonality six hybridomas continued to secrete nucleic acid binding antibodies and could be cultured in large volumes. This pattern of regression is not unexpected (Mosmann, T.R., unpublished observations). The experiments described below were performed on the medium obtained from these six monoclonal hybridomas. HEd 5-8 were obtained from the mice injected with 25μg LPS, and HEd 9 and 10 were derived from the mice injected with 50μg LPS.

By far the most probable classes of immunoglobulin produced by the hybridoma technology are IgM and IgG. These can be distinguished on the basis of their molecular weights (1,000,000 for IgM and 150,000 for IgG) using gel filtration on a Sephadex G200 column. Typical elution profiles are shown in Fig. 1 for media from clones HEd 9 and HEd 7. In the first case the antibodies were eluted in the excluded protein peak and thus HEd 9 is identified as an IgM, as is HEd 5. For HEd 7 on the other hand the antibodies were partially included on the column and thus HEd 7 is an IgG as are HEd 6, HEd 8 and HEd 10.

Before testing the specficity of the antibodies, serial dilutions of the various media were made in order to ascertain the greatest dilution which still gave rise to a maximum response. The results ranged from dilutions of 1 in 10 to 1 in 25 and the following SPRIA assays were performed at these

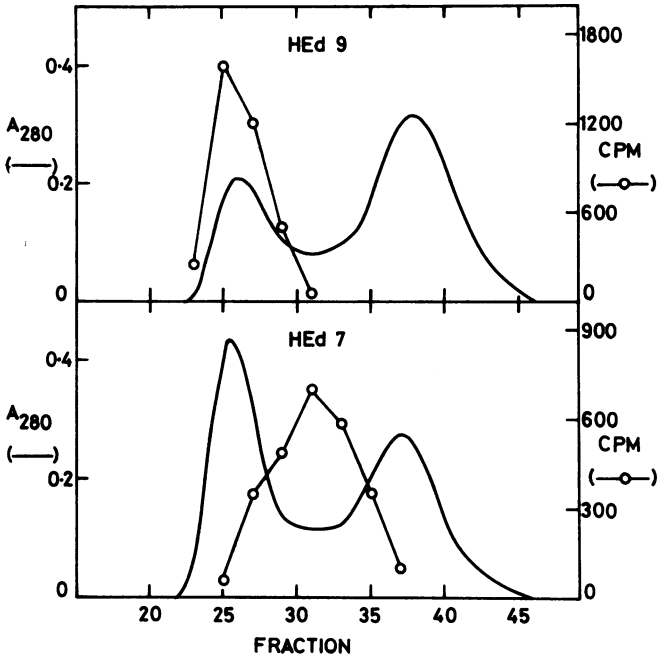

Figure 1. Gel exclusion chromatography of media from hybridomas (a) HEd 9 and (b) HEd 7 on a Sephadex G200 column. The fractions eluting from the column were monitored at 280 nm (———) and also tested with the SPRIA assay using (a) dT_n or (b) heat-denatured calf thymus DNA as the antigen (———o———). Bromophenol blue and blue dextran as molecular weight markers showed that the first protein peak was excluded while the second was largely included.

dilutions. The results were very reproducible (±20%) and in general only one measurement was performed for each antigen. A false positive was only recorded on one occasion. Since heat-denatured calf thymus DNA and dT_n were always run for comparative purposes they also served as a check on the internal consistency of the assay. The specific activity of various preparations of the I^{125} goat anti-mouse IgG was such that with heat-denatured calf thymus DNA or dT_n as antigen (see below) the CPM measured in the SPRIA assay were always in excess of 1,000 (usually 2,000-10,000) compared to a background (i.e. in the absence of added antigen) of less than 50 CPM.

Table I presents the results for the six hybridomas tested against the individual nucleic acids comprising groups A and B. Except for HEd 9 the preferred antigen is heat-denatured calf thymus DNA which is arbitrarily set at 100%. For HEd 9 the preferred antigen is dT_n and this is arbitrarily set at 100%. This confirms the original observation that the preferred antigen in all cases is a single-stranded DNA. Surprisingly, there is no detectable binding to single-stranded RNAs. This was confirmed using competition experiments in which the antibodies were premixed with 0.1 µg of ribosomal RNA before

Table I. Results of the SPRIA assay for the six monoclonal antibodies tested against various RNA and DNA polymers. All are normalized against heat-denatured calf thymus DNA at 100% except for HEd 9, where dT_n is used as the standard at 100%.

	HEd 5	HEd 6	HEd 7	HEd 8	HEd 9	HEd 10
Heat-Denatured Calf-Thymus DNA	100	100	100	100	<5	100
dT_n	<5	58	83	49	100	48
Ribosomal RNA	<5	<5	<5	<5	<5	<5
rU_n	<5	<5	<5	<5	<5	<5
Native Calf Thymus DNA	86	50	60	58	<5	37
$rA_n.dT_n$	<5	8	<5	22	<5	23
$dT_n.dA_n.dT_n$	22	32	<5	53	<5	50
$dA_n.dT_n$	<5	8	<5	15	<5	17
$d(TTG)_n.d(CAA)_n$	<5	<5	<5	<5	<5	<5
$d(TC)_n.d(GA)_n$	8	<5	<5	<5	<5	<5
Blank well	<5	<5	<5	<5	<5	<5

adding to the plates. Since approximately 1 ng of heat-denatured calf thymus DNA was bound to the plates this represents a hundred-fold excess of RNA. For HEd 8 and HEd 10 this competition resulted in an approximately 25% drop in the response to the SPRIA assay while for all other antibodies there was no significant change. Also shown in Table I is the interaction of the six types of antibody with three repeating-sequence duplex polymers. The small response shown by some of the antibodies to $dA_n \cdot dT_n$ may be due to binding to dT_n by opening up the duplex. In all cases the binding is barely detectable, if at all, which would tend to confirm the view that the antibodies prefer single-stranded DNAs. To further test this hypothesis the results for various single-stranded purine and pyrimidine DNAs are shown in Tables II and III

Table II. Results of the SPRIA assay for the six monoclonal antibodies tested against various polypurine DNAs.

	HEd 5	HEd 6	HEd 7	HEd 8	HEd 9	HEd 10
Heat-Denatured Calf-Thymus DNA	100	100	100	100	<5	100
dT_n	<5	58	83	49	100	48
dA_n	<5	<5	<5	<5	<5	<5
dI_n	<5	55	<5	<5	<5	<5
dG_n	7	17	<5	<5	<5	<5
$d(GAA)_n$	7	14	<5	8	<5	9
$d(GA)_n$	<5	<5	<5	<5	<5	<5
$d(GGA)_n$	<5	<5	<5	<5	<5	<5
$d(Gn^2A)_n$	<5	<5	<5	<5	<5	<5
$d(Gm^6A)_n$	<5	<5	<5	<5	<5	<5

Table III. Results of the SPRIA assay for the six monoclonal antibodies tested against various polypyrimidine DNAs.

	HEd 5	HEd 6	HEd 7	HEd 8	HEd 9	HEd 10
Heat-Denatured Calf Thymus DNA	100	100	100	100	<5	100
dT_n	<5	58	83	49	100	48
$d(TTC)_n$	<5	26	68	25	96	35
$d(TC)_n$	<5	66	39	67	21	64
$d(TCC)_n$	<5	59	<5	46	<5	70
dC_n	<5	<5	<5	<5	<5	<5

respectively. Surprisingly, except for HEd 6 binding to dI_n, the interaction with polypurine DNAs is limited. Recently it was shown (12) that repeating-sequence polypurines containing hypoxanthine or guanine form stable self-structures which are probably tetraplexes. However, dA_n and $d(Gm^6A)_n$ are truly single-stranded at neutral pH (12) and therefore the limited binding to polypurines cannot be interpreted simply as being due to the formation of DNA self-structure.

Repeating-sequence pyrimidine DNAs on the other hand, show significant binding to these antibodies except for HEd 5 (Table III). Surprisingly, in no case was there significant binding to dC_n but for HEd 6, HEd 8 and HEd 10 there was a strong interaction with $d(TCC)_n$. For HEd 7 and HEd 9 the results are presented graphically in Figure 2. It would appear that two consecutive thymines are required for a strong interaction. As far as we are aware, this is the first demonstration of sequence specificity in the interaction of antibodies with nucleic acid polymers, although specificity has previously been demonstrated with deoxyribodi- and tri-nucleotides (14,15).

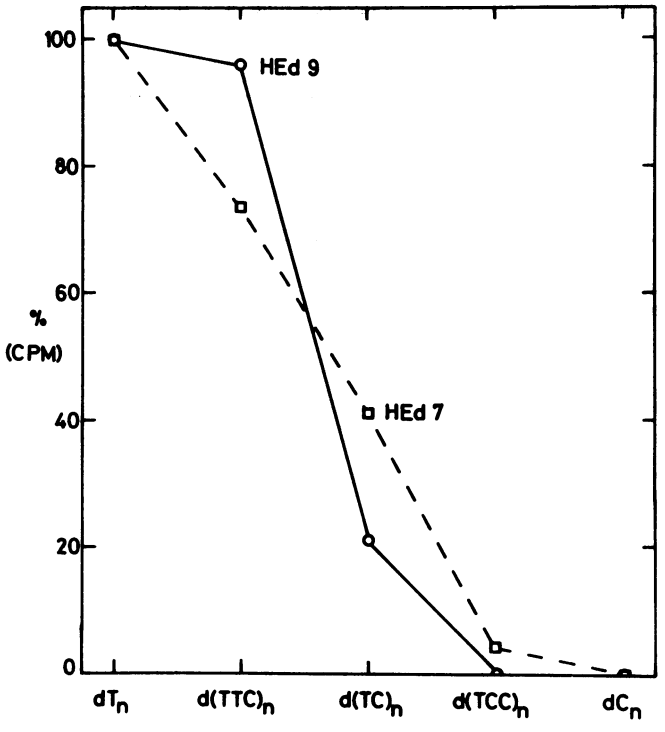

Figure 2. Effect of base composition on the binding of HEd 9 (○) and HEd 7 (□) to various single-stranded pyrimidine DNAs. The CPM from the SPRIA assay are shown as a percentage of that found for dT_n as the antigen.

The interaction of the antibodies with DNAs containing substituted pyrimidines is shown in Table IV. Except for HEd 5 (which doesn't bind to dT_n) the binding of the antibodies to dU_n is considerably weaker than to dT_n. This would suggest an important role for the 5 position of pyrimidines and this was further investigated with pyrimidine DNAs containing either bromine or iodine in the 5 position. Very surprisingly all halogenated pyrimidine DNAs (even $dBrC_n$) gave strong responses with all the antibodies (even HEd 5 which does not bind to any non-halogenated pyrimidine). Halogenation in itself is not sufficient for binding since the duplexes $d(BrUG)_n \cdot d(CA)_n$ and $dA_n \cdot dBrU_n$ give background readings.

Table IV. Results of the SPRIA assay for the six monoclonal antibodies tested against various DNAs containing alternating pyrimidine-purine sequences or substituted pyrimidines.

	HEd 5	HEd 6	HEd 7	HEd 8	HEd 9	HEd 10
Heat-Denatured Calf-Thymus DNA	100	100	100	100	<5	100
dT_n	<5	58	83	49	100	48
dU_n	<5	23	<5	9	<5	11
$dBrU_n$	108	113	99	78	95	103
dIU_n	107	144	191	99	111	107
$dBrC_n$	111	226	99	126	135	124
$d(BrUG)_n.d(CA)_n$	<5	<5	<5	<5	<5	<5
$dA_n.dBrU_n$	<5	<5	<5	<5	<5	<5
$d(AT)_n$	<5	<5	<5	6	<5	6
$d(TG)_n$	50	85	35	66	108	56
$d(CA)_n$	68	8	<5	<5	137	<5
$d(TG)_n.d(CA)_n$	<5	<5	<5	<5	<5	<5

Finally, in Table IV are shown the results for duplex and single-stranded alternating pyrimidine/purine DNAs. HEd 5 binds well to both $d(TG)_n$ and $d(CA)_n$ suggesting that in the absence of halogenated bases the preferred antigen contains alternating pyrimidines and purines since the interaction with virtually all other single-stranded DNAs is negligible (Tables I, II and III). Thus this is another example of sequence specificity. Whereas all the antibodies bind to some extent to $d(TG)_n$ and/or

$d(CA)_n$ the interaction with the duplex $d(TG)_n \cdot d(CA)_n$ is in all cases insignificant. This serves to emphasize the preference of these antibodies for single-stranded DNAs.

DISCUSSION

We have described the production of six hybridomas together with an investigation of the binding of their monoclonal antibodies to various nucleic acids. All of the six types of antibody bind exclusively to DNAs (with a preference for single-stranded DNAs) and there is no detectable interaction with RNAs. Two previous studies with hybridomas from autoimmune NZB/NZW mice produced monoclonal antibodies showing specificity for ribosomal RNA and duplex DNA (16,17). Thus it seems possible that the selection of monoclonal antibodies specific for single-stranded DNAs in this study may be an artefact of the initial screening procedure where mixed groups of antigens were used. However it is clear that this type of detailed analysis may be extremely useful in the study of the aetiology of autoimmune diseases.

Close scrutiny of the binding data for HEd 8 and HEd 10 show that their properties are very similar. For example, they are both positive for the same group of 16 antigens of the 33 different antigens tested. Moreover, preliminary isoelectric focusing experiments show that the heavy and light chains of HEd 8 and HEd 10 are not resolvable. Thus both hybridomas may be secreting identical antibodies, yet these two hybridomas are derived from different animals. Although these mice are syngeneic this result might suggest that there is a rather-limited set of potential autoimmune antibodies. Finally from the immunological point of view the discovery of both IgM and IgG classes of antibodies suggests that this autoimmune disease progresses through the same stages as other immune responses.

The patterns of specificity shown by the various antibodies are extremely difficult to interpret. For example HEd 9 in its interaction with pyrimidine DNAs appears to show a clear preference for two consecutive thymines (Fig. 2) and it does not bind significantly to dG_n, dA_n or dC_n. Yet it shows a

very strong response to both $d(TG)_n$ and to $d(CA)_n$. This complexity is not surprising since the antibody combining site could accommodate several bases, and these could contribute varying amounts to the binding energy. For example, phosphate and deoxyribose moieties could contribute a major part of the binding energy, and only a small extra contribution by base-specific interactions would be required. If this latter contribution could be provided by more than one base or combination of bases, the specificity patterns would be extremely complex. The binding of all six monoclonal antibodies is profoundly influenced by the base sequence of the single-stranded DNA antigen, and so all six are to some extent sequence-specific. The exact nature of the antigenic determinant, however, is probably best determined by direct structural (X-ray crystallographic) studies, or by inhibition of binding with short oligonucleotides of defined sequence. It is also unclear whether the preferred antigen has been identified in all cases. For example HEd 7, apparently binds best to heat-denatured calf thymus DNA, of all the antigens tested, suggesting that this DNA contains a sequence (the preferred binding site) which has not been tested individually. Another problem in the identification of the preferred antigen is that the number of bases being recognized may be as large as five or even more (18), and repeating sequence polymers of this length are not available. A summary of the hybridomas and their apparent specificities is shown in Table V.

All of the antibodies bound extremely well to $dBrU_n$, dIU_n and $dBrC_n$ even though there was no detectable binding of any of them to dC_n and only weak binding to dU_n. It seems possible that this strong binding to halogenated DNAs may be a non-specific effect due to the very polarizable nature of these polymers. Support for this view comes from the fact that the presence of these polymers on the PVC plates leads to a large increase in the background binding of the I^{125}goat anti-mouse IgG in the absence of added DNA binding antibody (data not shown). Thus the antibodies may be bound to the halogenated polymers at regions (e.g. the Fc portion) not directly involved with the antigen binding site. This requires further

Table V. Summary of the apparent specificities and class of antibodies from the six hybridomas. Halogenated polymers and native calf thymus DNA have not been included in the consideration of apparent specificities. See Tables 1 to 4 and refer to the text for further details.

Hybridoma (Laboratory Designation)	Apparent Specificity		Class of Antibody
	Preferred Antigen	Other Strongly-Bound Antigens	
HEd 5	Heat-denatured Calf Thymus	$d(TG)_n$, $d(CA)_n$	IgM
HEd 6	Heat-denatured Calf Thymus	dI_n, Single-stranded pyrimidine DNAs and $d(TG)_n$	IgG
HEd 7	Heat-denatured Calf Thymus	dT_n, $d(TTC)_n$	IgG
HEd 8	Heat-denatured Calf Thymus	Single-stranded pyrimidine DNAs and $d(TG)_n$	IgG
HEd 9	dT_n	$d(TTC)_n$, $d(TG)_n$, $d(CA)_n$	IgM
HEd 10	Heat-denatured Calf Thymus	Single-stranded pyrimidine DNAs and $d(TG)_n$	IgG

investigation.

Throughout this report it has been generally assumed that all antigens are bound to the PVC plates in approximately equal amounts. Some experiments with labelled antigens were performed to show that approximately 1 ng was bound though this test has not been possible with all antigens because of the lack of labelled polymers. There is little doubt however that there will be variations in the amount of antigen bound. For example heat-denatured calf thymus DNA has a complex secondary structure and is of a significantly higher molecular weight than the synthetic polymers. This may lead to more of it being bound to the plates which in turn might explain why for five of the six hybridomas this was the preferred antigen (excluding the halogenated polymers). However these potential problems with the SPRIA assay do not affect the general conclusions of

this study. First, the different types of antibodies in many cases complement one another. For example, it cannot be argued that the lack of a response of HEd 9 to $d(TCC)_n$ (Fig. 2) is due to lack of polymer being bound to the plate since HEd 6, HEd 8 and HEd 10 all give very significant results in the presence of this polymer. Second, in the case of the antibodies interacting with RNA and of HEd 9 with dC_n the negative result was directly confirmed using competition experiments. Finally other authors have used a similar SPRIA assay for antibodies binding to single-stranded and duplex DNAs (17,18); and in this study it was shown (by the fact that antibodies were bound) that the majority of the antigens tested were to some extent bound to the plates. Thus the binding of nucleic acids to the PVC plates is a widespread phenomenon and there is no good reason for believing that a particular nucleic acid antigen will not be bound.

The general conclusion of this study is that considerable specificity can be realized in the interaction of antibodies with nucleic acids. First the antibodies exhibit a structural specificity exemplified by their preference for single-stranded DNAs. Second they show an absolute requirement for a deoxyribose backbone since there is no detectable binding to RNA. Finally, they also demonstrate sequence specificity since they recognize differences in the nucleic acid bases. Therefore every basic feature of nucleic acid structure is being recognized simultaneously by the antibodies and consequently they have the potential for distinguishing any two nucleic acids however similar. It seems clear that monoclonal antibodies will find widespread use in many areas of nucleic acid research.

Abbreviations Used
HEd x - Hybridoma from Edmonton #x
PVC - Polyvinyl chloride
m^6A - 6-methylamino adenosine
n^2A - 2-amino adenosine
SPRIA - Solid Phase Radioimmunoassay
LPS - Lipopolysaccharide.

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