Antibodies against Tumor Cell Glycolipids and Proteins, but Not Mucins, Mediate Complement-Dependent Cytotoxicity[1]

Govind Ragupathi,* Nancy X. Liu,* Cristina Musselli,[2]* Shemeeakah Powell,* Kenneth Lloyd,† and Philip O. Livingston[3]*

One of several effector mechanisms thought to contribute to Ab efficacy against cancer is complement-dependent cytotoxicity (CDC). Serological analysis of a series of clinical trials conducted over a 10-year period suggested that six vaccines containing different glycolipids induced Abs mediating CDC whereas four vaccines containing carbohydrate or peptide epitopes carried almost exclusively by mucin molecules induced Abs that did not mediate CDC. To explore this further, we have now compared cell surface reactivity using flow cytometry assays (FACS), complement-fixing ability, and CDC activity of a panel of mAbs and immune sera from these trials on the same two tumor cell lines. Abs against glycolipids GM2, globo H and Lewis Y, protein KSA (epithelial cell adhesion molecule, also known as EpCAM) and mucin Ags Tn, sialylated Tn, Thomsen Friedenreich (TF), and MUC1 all reacted comparably by FACS with tumor cells expressing these Ags. Compared with the strong complement binding and CDC with Abs against glycolipids and KSA, complement binding was diminished with Abs against mucin Ags and no CDC was detected. A major difference between these two groups of Ags is proximity to the cell membrane. Glycolipids and globular glycoproteins extend less than 100 Å from the cell membrane while mucins extend up to 5000 Å. Although complement activation at sites remote from the cell membrane has long been known as a mechanism for resistance from complement lysis in bacteria, it is identified here for the first time as a factor which may contribute to resistance from CDC against cancer cells. *The Journal of Immunology,* **2005, 174: 5706–5712.**

Antibody-inducing vaccines based on defined protein and carbohydrate tumor-associated, cell surface Ags are being widely explored for the therapy of human cancers (1–10). Although the effectiveness of some of these vaccines has been demonstrated in a number of experimental model systems (11–14) and suggested in several clinical trials (1, 3, 15), the mechanism underlying their mode of action is uncertain. One possible effector mechanism would involve the complement-dependent cytotoxic (CDC)[4] activity of Abs of suitable class and subclass against the tumor cells. In clinical trials with vaccines against a variety of carbohydrate and protein Ags, we have noted that vaccines containing Ag covalently conjugated to keyhole limpet hemocyanin (KLH) plus immunological adjuvant QS-21 were optimal for inducing complement fixing IgM and IgG (IgG1 and IgG3) Abs in response to vaccination (2, 5–8, 10, 16–19).

In fact, in a number of clinical trials the Abs induced by these vaccines were shown to be capable of implementing CDC against tumor cells in vitro (5, 6, 8–10, 17, 18). In contrast, in a number of similar trials with other Ags, the Abs induced were found to have comparable levels of IgM Ab reactivity by IgM FACS but to be incapable of lysing target cells by CDC (2, 7, 17, 19). Closer inspection of these results (summarized in Table I) showed that the targets for effective CDC were glycolipids (e.g., GM2, GD2, GD3,[5] fucosyl GM1, globo H, or Lewis Y (Le^Y)) whereas those in which no lysis was observed were carbohydrate (e.g., Thomsen Friedenreich (TF), Tn, sialylated Tn (sTn)) or peptide (e.g., MUC1) epitopes carried by mucin molecules.

It was unclear whether this dichotomy was a result of the properties of the induced Abs (i.e., class and effector functions), the multiple different target cells used, or the nature of the target Ags. To resolve this, we compared the cell surface reactivity (assayed by FACS), complement-fixing ability (using the immune adherence (IA) assay and anti-C3b and C5b-9 FACS) and the CDC activity of a panel of mAbs and representative immune sera on the same two tumor cell lines.

Materials and Methods

Cell lines

The human colon cancer cell line LSC (expressing KSA, sTn, Tn, and Le^Y) (20) and the human breast cancer cell line MCF-7 (expressing GM2, globo H, Le^Y, sTn, TF, and MUC1) were obtained from Dr. S. H. Itzkowitz (Mount Sinai Medical Center, New York, NY) and H. D. Soule (Karmanos Cancer Institute, Detroit, MI), respectively. LSC cells were grown in MEM with 10% FBS and 1% glutamine. MCF-7 cells were grown in DMEM with 10% FBS and 1% glutamine.

Monoclonal Abs

mAb CC49 and CC102 against sTn were provided by Dr. J. Schlom (National Cancer Institute, Bethesda, MD); B239.1, against Tn, and 49H.8, against TF, were provided by Dr. R. Koganty (Biomira, Edmonton, Alberta, Canada); HBTn1 and 5F4, also against Tn, were purchased from

*Department of Medicine and †Immunology Program, Memorial Sloan-Kettering Cancer Center, New York City, NY 10021

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[2] Current address: Antigenics, 3 Forbes Road, Lexington, MA 02421.

[3] Address correspondence and reprint requests to Dr. Philip O. Livingston, Department of Medicine, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York City, NY 10021.

[4] Abbreviations used in this paper: CDC, complement-dependent cytotoxicity; KLH, keyhole limpet hemocyanin; Le^Y, Lewis Y Ag; TF, Thomsen Friedenreich Ag; sTn, sialylated Tn; IA, immune adherence; KSA, epithelial cell adhesion molecule (also known as EpCAM); HSA, human serum albumin; (c), cluster or trimer; DAF, decay-accelerating factor; MFI, mean fluorescence intensity; MAC, membrane attack complex; ADCC, Ab-dependent cellular cytotoxicity.

[5] GD2, GM2, and GD3 are used in accordance with the abbreviated ganglioside nomenclature of Svennerholm (58).

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DAKO; MBr1, against globo H, by Dr. M. I. Colnaghi (Istituto Nazionale per lo Studio e la Cura dei Tumori, Milan, Italy); BR96 and S193, against Le^Y, were provided by Dr. I. Hellstrom (Bristol-Meyers Squibb Pharmaceutical Research Institute, Seattle, WA) and Dr. G. Ritter (Ludwig Institute at Memorial Sloan-Kettering Cancer Center (MSKCC), New York, NY) respectively; 696, against GM2, by Dr. N. Hanai (Kyowa Hakko Kogyo, Tokyo, Japan); GA733, against KSA, by Dr. D. Herlyn (Wistar Institute, Philadelphia, PA). mAbs against MUC1 were C595, purchased from Serotec, 789 and NCRC11 provided by Dr. G. Denton (Queens Medical Center, Nottingham, U.K.), HuHMFG1 purchased from Antisoma Research Laboratories (London, U.K.), and VK5 provided by Dr. K. Lloyd (MSKCC, New York, NY). The Ab class, subclass, and references for these mAbs are listed in Table III.

Immune sera

Immune sera from patients vaccinated with sTn trimers or clusters (c), Tn(c), TF(c), MUC1, Le^Y, GM2, or globo H Ags conjugated to KLH were available from our clinical trials (2, 5–8, 10, 17–19). They were selected because these Ags are expressed by cell lines MCF7 and LSC. Pre- and peak titer postimmunization sera of representative patients were selected. Immune human sera against KSA were not available as we have not yet been able to induce relevant anti-KSA Abs.

Serological assays

ELISA were performed against purified glycolipids and mucins using anti-IgM and anti-IgG secondary Abs as previously described (2, 6). To control for nonspecific binding, immune sera were also tested on plates that were processed identically, but to which no Ag had been added, and the reading was subtracted from the value obtained in the presence of the Ag. Titer was defined as the highest dilution yielding a corrected optical density of ≥ 0.1.

Flow cytometry (FACS)

Assays were performed with single-cell suspensions of LSC and MCF-7 cells as previously described (5, 6). Patient pre- and posttreatment sera were used undiluted, 20 μl per sample, and tested together. The percentage of positive cells for pretreatment sera was set at 10%. The concentrations of mAbs used were the highest dilutions yielding the highest reactivity. They were mAb 696 at 0.6 μg/ml, GA733 at 1.8 μg/ml, VK-5 at 8 μg/ml, MBr1 at 4 μg/ml, HuHMFG1 at 10 μg/ml, 789 at 4 μg/ml, NCRC11 at 10 μg/ml, C595 at 10 μg/ml, CC49 at 0.8 μg/ml, B239.1 at 0.5 μg/ml, 5F4 at 100 μg/ml, HBTn1 supernatant at 1/4 dilution, B239.1 at 0.5 μg/ml, 49H.8 at 4 μg/ml, S193 at 0.5 μg/ml, BR96 at 4 μg/ml, and CC102 supernatant at 1/4 dilution. FITC-labeled goat anti-human IgG (Zymed Laboratories) or anti-human IgM or goat anti-mouse IgG or anti-mouse IgM (Southern Biotechnology Associates) were diluted 1/25, and used at 20 μl per well. Biotinylated murine mAbs against C3b, C6, C5b-9, and Bb used at 25 μl/tube were purchased from Quidel. FITC-labeled streptavidin from DAKO was used at a dilution of 1/100, 25 μl per tube. For inhibition studies, sera were first incubated for 30 min at 4°C with 10 μg of the immunizing or irrelevant (GD3) Ag as indicated in Table III before addition to the cell targets.

IA assays

IA assays were performed to determine complement binding of the mAb and patient sera against target cell lines as demonstrated by RBC rosetting (21). The assay is based on receptors for complement (C3) expressed at the cell surface of human RBC. Cells were plated in wells of 60-well Terasaki plates (Nalge Nunc International) at a concentration of 1000 cells/well and incubated for 32 h at 37°C in a 5% CO_2 atmosphere. Patient sera were added at a starting dilution of 1/10 and then serially diluted 1/2. mAbs were used at the concentrations discussed above and then serially diluted 1/1. Cells with patient sera or mAb were incubated at 4°C for 1 h. Human O^+ type RBC at a dilution of 1/5 and guinea pig complement at a dilution of 1/60 were then added to the wells. The plate was then incubated at 37°C for 30 min, washed, and examined under a light microscope. Cells were scored as positive when they were at least 50% covered with red cells.

Complement-dependent cytotoxicity

CDC was assayed on the LSC and MCF-7 cell lines using a 2-h ^{51}Cr release assay as previously described with human complement and pre- and posttreatment sera at dilutions of 1/4 and 1/100 (5, 6), or with mAbs at the dilutions described above. Approximately 10^7 cells were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ (New England Nuclear) in 3% human serum albumin (HSA) for 2 h at 37°C, shaking every 15 min. The cells were washed four times and brought to a concentration of 10^6 live cells/ml. Fifty microliters of labeled cells were mixed with 50 μl of undiluted pre- or postvaccination serum or with medium alone in 96-well, round-bottom plates (Corning) and incubated at 4°C on a shaker for 45 min. Human complement (Sigma Diagnostics) diluted 1/5 with 3% HSA was added, at 100 μl/well, and incubated at 37°C for 2 h. The plates were spun at 100 $\times$ *g* for 3 min, and an aliquot of 100 μl of supernatant from each well was read by a gamma counter to determine the amount of ^{51}Cr released. All samples were performed in triplicate and included control wells for maximum release and for spontaneous release in the absence of complement. Maximum release was the amount released by target cells after a 2-h incubation with 1% Triton X-100 (Sigma Diagnostics) and 100 μl of human complement. Spontaneous release (the amount released by target cells incubated with complement alone) was subtracted from both experimental and maximal release values. Specific release was equal to corrected experimental release divided by corrected maximal release: specific release (%) = (experimental release − spontaneous release)/)maximum release − spontaneous release).

Sera were considered positive by ELISA if the titer of reactivity was at least 1:40, by FACS if the percent of positive cells tripled (>30%) in posttreatment sera compared with pretreatment sera, and by CDC assay if there was 15% or more specific release. Results obtained with mAbs or immune sera against Ags expressed on mucins vs results obtained against other glycoproteins and glycolipids were compared using the χ^2 test.

Results

Binding and lytic activity of immune sera from vaccinated patients

The results of a series of previously reported clinical trials of conjugate vaccines against glycolipid and mucin Ags were retabulated to determine how many patients with clear FACS reactivity against the selected target cells had strong CDC (see Table I). Of patients vaccinated with glycolipid Ags, 48 had strong FACS and 39 had strong CDC, compared with the 42 patients vaccinated with mucin Ags who had strong FACS of whom not one had strong CDC ($p < 0.001$). In all cases, the Abs induced in patients have been IgM and IgG1 or IgG3 (6, 8, 9, 17, 23–25). These published results are consistent with our overall experience: 1) of the sera from >100 patients vaccinated with glycolipids and analyzed for FACS and CDC reactivity, the great majority of patients had both strong FACS reactivity and strong CDC and 2) of the >100 patients vaccinated with Ags expressed on mucins and analyzed for FACS and CDC, the great majority of patients have had strong FACS reactivity and only a single patient has had detectable CDC.

To determine whether the results in Table I and our overall experience could have been due to the use of different target cell lines, the long intervals between some of the serological analysis or to other variables related to comparing the results of assays performed at different times by different investigators in our laboratory, we repeated a portion of the serological analysis. All available sera from the trials in Table I that involved immunization against Ags expressed by the breast cancer cell line MCF7 and that yielded >30% positive MCF7 cells by anti-IgM FACS, were included (see Table II). Of the 42 patients vaccinated with glycolipid Ags who had >30% positive MCF7 cells by anti-IgM FACS, 28 had strong CDC. Of the 31 patients vaccinated with mucin Ags who had >30% positive MCF7 cells by anti-IgM FACS, not one had detectable CDC ($p < 0.001$).

Binding and lytic activities of mAbs

The two cell lines MCF-7 and LSC were selected for their expression of a range of glycolipid, protein, and mucin Ags. We obtained 16 mAbs of the proper specificity and complement fixing class and subclass for testing. The cell surface reactivity of these 15 murine (all IgM, IgG2a or IgG3) and 1 human (IgG1) mAbs against the appropriate target cell lines were tested by FACS (Table III). All mAbs tested strongly and comparably positive with their selected target cells. IA assays performed on the target cell lines with the same mAbs showed positive complement binding with 50–90% rosettes with all mAbs, though overall the levels of IA reactivity

Table I. *CDC is seen in sera from patients vaccinated with glycolipid Ags but not those vaccinated with mucin Ags (results from published studies)*[a]

Vaccine KLH-Conjugate Plus QS21	Target Cell Line	No. Patients in Trial	No. Patients with >30% Positive Cells (IgM FACS)	Median % Positive Cells (IgM FACS)	No. Patients with >20% CDC	Ref.
Glycolipid Ags						
FUC GM1	H146	10	9	67	9	5
GM2	SK-MEL 173, MGH-U3	13	12	66	11	43
		27	21	51	17	22
GD2L	NMB-7	12	7	44	4	59
GD3L	SK-MEL-28	6	3	36	2	8
Globo H	MCF7	46	27	61	19	7, 23
Le^Y	OVCAR-3	24	8	45	6	10
Mucin Ags						
MUC1[b]	MCF7	9	7	63	0[c]	7
MUC1G[d]	MCF7	22	18	75	0	24
sTn(c)	LSC	21	17	78	1	5,25
Tn(c)	DU145	5	4	54	0	60
Tn(c)	LSC	15	5	34	0	60
TF	DU145	10	5	51	0	61

[a] Meta-analysis p value for 68 of 88 patients vaccinated with strong FACS who had CDC compared to 1 of 56 patients vaccinated with mucin Ags with strong FACS who had CDC: $p < 0.001$.
[b] Thirty-two amino acid MUC1 peptide.
[c] Zero (0) means no patient showed a significant increase in posttreatment CDC over pretreatment CDC.
[d] One hundred and six amino acid MUC1 peptide glycosylated with Tn epitopes *O*-linked at 26 sites. Reactivity is against both MUC1 and Tn.

were higher with mAbs against glycolipid Ags than mucin Ags. CDC performed on the target cell lines with five mAbs against GM2, globo H, Le^Y, and KSA was positive with all five mAbs, yielding complement-dependent lysis of between 44.2 and 97.5%. In contrast, not one of 11 mAbs against the mucin Ags Tn, sTn, TF, and MUC1 showed significant complement-dependent lysis (Table II, $p < 0.001$).

Activation of complement factor C5b-9 at the tumor cell surface

To help determine the basis for the lack of CDC with mucin Ags as targets, we repeated the flow cytometry studies in the presence of human complement using second Abs against murine or human Ig, and against human C3b or C5b-9. mAbs against the glycolipid Ags Ley, GM2, and globo H and the mucin Ags MUC1, sTn, and Tn had comparable cell surface Ig binding. For Abs against the glycolipid Ags this resulted in potent activation of C3b as well as consistent activation of C5b-9 at the cell surface (see Table IV) and CDC. However, for mAbs against mucin Ags that induce comparable Ig cell surface binding by FACS, activation of complement as indicated by binding of C3b was consistent but diminished and there was no binding of C5b-9, and no evidence of CDC.

Discussion

CDC may be one of the mechanisms whereby Abs against tumor Ags eradicate circulating tumor cells and micrometastases. Effective CDC depends partly on the class and subclass of the Abs and their differing abilities to fix complement. Thus, with IgM and IgG Abs of similar affinity, IgM Abs are particularly potent in CDC, whereas only certain subclasses of IgG (IgG1 and IgG3 in humans, IgG2a and IgG3 in mice) can efficiently activate complement. The particular effectiveness of IgM with repetitive or clustered epitopes may be due to the pentameric nature of the IgM structure with its resulting increased avidity and complement-fixing activity. Also, not all tumors or tumor cell lines are equally sensitive to CDC. Some tumor cells and cell lines are known to express a range of complement-inactivating factors at the cell surface such as decay-activating factor (DAF), which interferes with assembly of C3

Table II. *CDC against MCF7 is seen with sera from patients vaccinated with glycolipid Ags but not those vaccinated with mucin Ags (results of retesting positive sera from Table 1)*[a]

Vaccine KLH-Conjugate Plus QS21	Target Cell Line	No. Patients in Trial	No. Patients with >30% Positive Cells by IgM FACS	IgM FACS Median % Positive Cells/MFI of Responders		No. Patients with >20% CDC Increase
				Pre	Post	
Glycolipid Ags						
Le^Y	MCF7	9	2	10/16	48/28	2
GM2	MCF7	16	12	10/46	43/118	7
Globo H	MCF7	27	19	10/99	51/230	8
Mucin Ags						
MUC1[b]	MCF7	9	7	10/38	65/91	0[c]
MUC1G[d]	MCF7	18	12	10/21	43/57	0
sTn(c)	MCF7	27	11	10/31	48/85	0
TF(c)	MCF7	5	2	10/34	60/145	0

[a] All available sera from the trials in Table I that involved immunization against Ags expressed by the breast cancer cell line MCF7 and that had yielded >30% positive MCF7 cells by FACS were retested. Value of p for 17 of 33 patients vaccinated with glycolipid Ags with strong FACS who had CDC compared to 0 of 32 patients vaccinated with mucin Ags with strong FACS and CDC: <0.001.
[b] Thirty-two amino acid MUC1 peptide.
[c] No patient showed a significant increase in posttreatment CDC over pretreatment CDC.
[d] Thirty-two amino acid MUC1 peptide glycosylated with Tn epitopes *o*-linked at four or six sites. Reactivity is against both MUC1 and Tn.

Table III. *Comparison of FACS, IA, and CDC reactivity with tumor cells of mAbs against antigenic epitopes on glycolipids, a globular protein, and mucins*[a]

	mAb	Ref.	mAb Subclass	Target Cell Line	FACS % Positive Cells	IA % Rosetted	CDC % Lysis
Glycolipid Ags							
GM2	696	26	IgM	MCF-7	97	80	44
Globo H	MBr1	27	IgM	MCF-7	96	80	97
Le^Y	S193	28	IgG3	LSC	97	80	69
				MCF7	80	60	70
	BR96	29	IgG3	LSC	81	90	83
				MCF7	88	80	80
Globular Protein Ags							
KSA	GA733	30	IgG2a	LSC	99	70	75
Mucin Ags							
sTn	CC102	31	IgM	LSC	89	70	0
	CC49	31	IgG1	MCF7	90	50	0
Tn	B239.1	32	IgM	LSC	99	90	5
	HBTn1	33	IgM	MCF7	87	70	0
	5F4	34	IgM	MCF7	98	40	0
TF	49H.8	35	IgM	MCF7	80	50	0.7
MUC1	VK-5	–[b]	IgG2a	MCF7	88	50	0.2
	NCRC11	36	IgM	MCF7	84	50	0
	789	37	IgM	MCF7	91	50	0
	C595	38	IgG3	MCF7	84	60	6
	HuHMFG1	39	IgG1[c]	MCF7	45	50	2

[a] Value of p for comparison of five of five mAbs with CDC against glycolipid and globular protein Ags to 0 of 11 mAbs against mucin Ags is <0.001.
[b] Unpublished results (K. O. Lloyd and V. Kudryashov); this mAb reacts with the PDTRPA sequence within the MUC1 core.
[c] Humanized mAb (human IgG1 Fc).

(40), and CD59, which inhibits complement membrane attack complex formation (41, 42).

In the present study we have identified a new factor in determining the effectiveness of CDC against tumor cells which may be quite separate from Ab class/subclass and complement-fixing considerations, or tumor cell resistance mechanisms. It has to do with the biochemical and biophysical nature of the target Ag. The key finding is that tumor cells can be readily lysed by Ab and complement when the target epitopes are on glycolipids or globular proteins, but comparable lysis is not seen when the target epitopes are on mucins. This finding originated in our observations in a series of small clinical trials with vaccines against these two classes of Ags (2, 5–10, 16–19, 22–24, 43, 59), and is supported by a new analysis of these results. To exclude the possibility that this dichotomy was a consequence of the different target cells used or several other artifactual possibilities, all available high titer sera against Ags expressed on MCF7 cells from these trials were retested simultaneously on MCF7. The result was the same, most sera against glycolipid Ags but none of the sera against mucin Ags induced CDC of MCF7 cells. The subclasses of the IgG Abs induced against the glycolipid and mucin Ags have been shown to be the same, the two complement-activating subclasses IgG1 and 3 (GM2 (43), globo H (23), MUC1 (7, 24), sTn (25)), but the results were also the same when comparison was restricted to sera with strong IgM reactivity against MCF7. Results in the reanalysis and the retesting were both highly significant. To exclude the possibility that this finding was due to idiosyncrasies related to Abs induced by the conjugate vaccines in these patients, we obtained 16 mAbs with known complement-fixing ability against the same Ags. Five mAbs against glycolipids (GM2, globo H, and LeY) or globular proteins (KSA) induced strong CDC while 11 mAbs against mucin Ags that are not expressed as glycolipids (Tn, sTn, TF, and MUC1) induced no detectable CDC ($p < 0.001$). Because the class, subclass, and FACS results (percent-positive cells and mean fluorescence intensity (MFI)) for most of the mAbs in these two groups were comparable (see Table III), only MCF7 and LSC cells were used as targets and the assays were done simultaneously, this strongly implicated the nature of the Ags as the determining factor.

Table IV. *mAbs against glycolipid but not mucin Ags activate complement C5b-9 on the tumor cell surface*

	mAb		Specificity of Second Ab (% positive cells/MFI)				
Ag	Name	Class	Anti-Ig	Anti-C3b	Anti-C5b-9	Anti-Bb[a]	CDC (%)
Glycolipid Ags							
Le^Y	S193	IgG3	99/835	95/1130	65/81	7/19	78
GM^2	Pgnx	IgM	93/993	97/1107	58/98	6/22	71
Globo H	VK9	IgG	97/372	94/906	24/36	4/14	40
Mucin Ags							
MUC1	HuHMFG1	HuIgG1	93/458	20/52	6/22	5/15	3
sTn	CC102	IgM	97/869	60/82	1/20	6/17	0
Tn	HB-Tn.1	IgM	99/1134	73/117	7/26	5/16	4

[a] Negative control.

There have been three reports describing CDC against mucin glycoproteins on epithelial cancer cells. The first demonstrated CDC against a breast cancer cell line with sera from breast cancer patients immunized with a MUC1 vaccine. Although the chromium release assay was similar to ours, there were two differences: rabbit, not human, complement and round-, not flat-, bottom plates were used (62). We have reported previously that Abs induced against polysialic acid (another Ag that extends a great distance from the cell surface) were not able to induce CDC against tumor cells (65). These Abs were able to lyse polysialic acid positive bacteria in the presence of rodent complement but not in the presence of human complement. The basis for the differential reactivity of rodent complement over human complement is unclear. The second found no CDC against DU145 prostate cancer cells with two mAbs unless DAF was first blocked, in which case a low level of CDC was seen. In the experiments described here, we demonstrated strong CDC with Abs against glycolipids on the same cells that could not be lysed with Abs against mucin Ags (63). If DAF has a role in protection from CDC mediated by mucin Ags, it must be quantitatively if not qualitatively different than for glycolipid Ags. This again supports the conclusion that there is a difference in sensitivity to CDC between Ags expressed on mucins and Ags expressed on other glycoproteins or glycolipids. The third report found CDC using mAbs against Ags that were expressed both on mucins and glycolipids (64). It is likely that the Ags responsible for CDC were expressed on other glycoproteins or glycolipids such as globo H, Le^Y, or other of the many Ags that can be expressed on both glycolipids and glycoproteins. MUC1, Tn, sTn, and TF, in contrast, are not known to be expressed on glycolipids, making the distinction clear.

The most obvious difference between the glycolipid and globular protein Ags on the one hand, and mucin Ags on the other, is the molecular architecture and profile of the Ags as they are expressed on the cell surface. Glycosphingolipids with four or five sugar residues are relatively small molecules, residing close (within 50 angstroms) to the cell surface lipid bilayer in which their ceramide moieties are embedded. Globular proteins such as KSA, are somewhat larger in size (40 kDa) but would still not extend >100 Å above the cell membrane (44). Mucins, in contrast, are very large molecules with molecular masses in the 200-2000 kDa range, 50–80% consisting of the highly glycosylated tandem repeat portion. Furthermore, because of the high degree of glycosylation of the tandem repeat peptide core, this portion would be expected to assume a rigid, rod-like structure extending far above the cell surface (up to 5000 Å for MUC-1) and even above the glycocalyx characteristic of epithelial cells (44, 45). Experimental support for this structure comes from nuclear magnetic resonance and other physical measurements on the MUC-1 and leukosialin (CD43) mucins (45, 46). Both studies support an extended peptide backbone for the mucin and the study on leukosialin shows that *O*-glycosylation of serine and threonine residues within the peptide sequence is what ensures a stable elongated structure (46). The most likely explanation for lack of cytotoxicity in the face of effective complement activation is that with epitopes on mucins the great majority of C′-fixation occurs at sites too far removed from the plasma membrane for the complement components to be effective in cell lysis.

Complement activation through Ag-Ab complexes begins with the binding of the C1 complement component. This initiates a cascade of enzyme activities resulting in binding of C3b and eventually insertion of the C5b-9 protein complement membrane attack complex (MAC) into the cell membranes to form pores. Dimensions of the MAC are 100 × 150 Å (47). The m.w. of the MUC1 C-terminal extracellular subunit and flanking sequence are in excess of 100 kDa (37), making it likely that the MUC1 tandem repeat portion begins 100 Å or more from the cell membrane. This is where the target MUC1 peptide and carbohydrates begin. If complement activation occurs at sites more distant than 100 Å from the cell membrane, the MAC would not form or if formed would not reach the cell membrane and a number of serum proteins would quickly inactivate the forming MAC (47). Complement-inactivating factors cannot explain these findings because CDC was readily induced by Abs against glycolipids in the same experiments and same two cell lines that could not be lysed by mucin Abs.

Although resistance to complement-dependent lysis as a consequence of complement activation at sites remote from the cell membrane has not been previously described for cancer cells, it has long been known as a source of bacterial resistance to CDC (reviewed in Refs. 48 and 49). Comparing *Salmonella minnesota* with smooth (long LPS chains) and rough phenotype (short LPS chains), C3 binding and C5b-9 consumption after Ab binding were similar or increased with the smooth phenotypes but this was associated with "failure of complexes to bind hydrophobically in the outer membrane" of these smooth variants (50). Similar findings have been described for *Salmonella montevideo* (51) and *Pseudomonas aeruginosa* where serum resistance is associated with long LPS chains. Complement activation occurs but there is "failure of the assembled terminal complement complex C5b-9 to insert stably into the outer membrane" (52). Comparing serum-sensitive (rough) and serum-resistant (smooth) strains of *Klebsiella pneumoniae*, both use and activate complement (delete it from the media) and bind C3b (though smooth strains bound less C3b than rough strains), but only the sensitive (rough) strains were able to bind C5b-9 (53). Binding of C3b was shown by immunogold electron microscopy to occur farther from the cell surface of smooth cell strains than rough strains. Merino et al. (53) conclude that: "The reasons for this resistance are that C3 binds far from the cell membrane and that the lytic final complex C5b-9 (membrane attack complex) is not formed. Isogenic rough mutants are serum sensitive because they bind C3b closer to the cell membrane and the lytic complex C5b-9 is formed" (53). The same phenomenon has been described for a variety of Gram-positive bacteria that have a thick peptidoglycan layer at the cell surface (54). Despite the resistance to complement-mediated cytotoxicity in each of these settings, bacterial growth of even complement-resistant strains is generally controlled by the host as a consequence of other mechanisms including complement-mediated opsonization, inflammation, and phagocytosis.

We found that for Abs against glycolipids, cell surface binding, C3b and C5b-9 expression, and CDC were all strong, while for Abs against mucin Ags, cell surface binding was equally strong but resulted in diminished C3b and totally absent C5b-9 binding or CDC. This is similar to the results with smooth and rough *Klebsiella* described above. The basis for the relative decrease in C3b binding to Abs against smooth strains of *Klebsiella* and against mucin Ags remains unclear but may result from the differential pathways for C3b breakdown when C3b becomes attached to the cell surface. It must be emphasized that although we show here that mucins are poor targets for complement-mediated lysis of tumor cells, studies have shown that induction of Abs against either glycolipid or mucin Ags results in protection from tumor recurrence in several different preclinical mouse models (11–14). Also, Abs against either glycolipid or mucin epitopes correlate with a more favorable prognosis in patients (1, 3, 15, 16, 55, 56). It does not appear that the inability of Abs against mucin Ags to induce complement-mediated lysis is necessarily detrimental to the antitumor response. Consequently, inflammation, opsonization, and

Ab-dependent cellular cytotoxicity (ADCC) but not CDC are likely mechanisms for the prolonged survival seen in the preclinical experiments targeting mucin Ags and suggested in the clinical trials with passively administered and actively induced Abs against mucin Ags. With regard to bacterial infections, this is supported by the severe consequences of hereditary deficiency states involving either the classical or alternate complement pathways and the comparatively trivial consequences to deficiencies of the MAC (49).

In light of the relative inability of Abs against Ags on mucins to bind C3b and in most cases their absolute inability to bind C5b-9 or to mediate CDC, it will be important to compare other effector mechanisms of Abs against mucin and non-mucin cancer Ags. It may be that other Ab-mediated mechanisms such as ADCC, opsonization of tumor cells by leukocytes, induction of apoptosis, and blocking of tumor cell invasion or metastasis also differ depending on the biochemical and biophysical nature of the Ag. To date, studies with sera from some of the same MUC1 immunized patients have shown that at least with regard to Abs against MUC1 peptide, ADCC can be mediated by postimmunization sera (57).

Disclosures

P. Livingston is a paid consultant and stockholder in Progenics Pharmaceuticals which has licensed the GM2-KLH, GD2-KLH, and GD3-KLH vaccines.

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