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Intratumor immunologic heterogeneity

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Summary

Tumor cells express a great variety of antigens including tumor specific transplantation antigens, tumor-associated antigens, differentiation antigens, histocompatibility antigens, lectin-binding sites and receptors for natural killer cells and natural antibodies. These antigens are distributed unevenly on tumor subpopulations and each subpopulation may induce different immune responses to the same determinant. Intratumor immunologic heterogeneity arises early in cancer, possibly during preneoplasia, and exists throughout the course of progression. Metastatic subpopulations are not generally less antigenic than subpopulations within primary tumors. Different arrays of antigenic determinants are displayed by subpopulations but variability in cell surface expression of a single determinant is also a fundamental type of immunologic heterogeneity. Antigenic specificity patterns commonly reveal one-way cross-reactions between tumor subpopulations. One-way cross-reactions might occur due to quantitative differences, cell-cycle variations, modulation, masking, H-2 expression and restriction phenomena, or alteration in the carbohydrate side-chains of glycoproteins. Interactions which occur when subpopulations co-exist may alter immune responses so that the response to the mixture is not the sum of the responses to the individual subpopulations. It is suggested that the exploitation of the mechanisms involved in immunologic heterogeneity may lead to new therapeutic approaches and that the great diversity of determinants expressed by tumor cells could lead to development of multivalent panel of monoclonal antisera which, acting synergistically, could preferentially lyse tumor cells.

Introduction

Immunotherapy as a means of cancer treatment was envisioned long before tumor specific antigens were described (1). The advent of syngeneic strains of experimental animals allowed the description of the laws of tissue transplantation (2) and the demonstration of tumor specific transplantation antigens or TSTAs (3). Armed with inbred animals and knowledge of the laws of transplantation, many investigators began to study tumor specific antigens and numerous assays to detect cellular and humoral responses were devised. Initial results with chemically- or virally-induced tumors were encouraging. However, spontaneous tumors, i.e., those induced by no known agent, are seldom detectably antigenic (4, 5), but demonstration of antigenicity is 'dependent upon the sensitivity of the tests' (6) so spontaneous

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tumors may in fact be very weakly immunogenic. In any event, the promise of immunotherapy as a treatment remains viable.

It has gradually become apparent that tumor cells exhibit, not just one, but several antigens capable of eliciting responses in syngeneic animals. In addition to the private or unique TSTAs, common viral antigens (7, 8) and oncofetal, often tissue specific, antigens have been described (9). For years, the presence of cross-reactive, tumor-associated transplantation antigens was taken as evidence that tumors were viral-induced, although these tumors could also have TSTAs in addition to tumor-associated transplantation antigens (10, 11); Chemical-carcinogen-induced tumors were thought to be antigenically distinct.

Recently, it has been shown that, depending on both the method of immunization and the assay for antigenicity, three classes of tumor antigens may be defined. Two methylcholanthrene (MCA)-induced tumors were found to express cross-reactive, tumor-associated transplantation antigens as well as classical TSTAs (12). TSTAs were demonstrated after syngeneic mice were immunized by excision of transplanted primary tumors and challenged with relatively high doses (5×10^5 cells); Cross-reactive transplantation resistance was detectable if these immunized animals were challenged with a low dose (1×10^4 cells). Immunization with four weekly injections of irradiated tumor cells also resulted in cross-reactive resistance to tumor challenge. Roberts et al. (13) suggested that tumors with related non-viral etiology, e.g., ultraviolet (UV)-induced or MCA-induced, share a second class of antigens which they called tumor-associated transplantation antigens which function in vivo as cross-reactive transplantation antigens but only in hyperimmunized mice (as opposed to a single immunization to induce immunity to TSTAs). A third class of antigens, shared by UV-induced and MCA-induced tumors and which Roberts et al. (13) called tumor-associated antigens, generate cytotoxic T lymphocytes (CTLs) in vitro and are recognized by CTLs in vivo but induce suppressor T-cells in vivo. Tumor cells also express a spectrum of cell surface determinants found on normal cells such as histocompatibility antigens, differentiation antigens and lectin-binding sites.

In the last few years it has become evident that all of these antigens and surface determinants are not uniformly distributed within tumors but, instead, individual subpopulations of a tumor may differentially express antigens of each class. This intratumor immunologic heterogeneity poses a new challenge to the development of effective immunotherapy, as well as reliable immunodiagnostic methods.

Immunologic heterogeneity of primary neoplasma

The heterogeneous nature of cancer has received much attention recently (14–17). Intra-tumor heterogeneity for many phenotypes exists; A catalogue of phenotypes for which tumor subpopulations are heterogeneous is probably limited only by the number of phenotypes studied. Antigenicity is one such phenotype for which intra-tumor heterogeneity was demonstrated as early as 1970 (18), but intratumor immunologic heterogeneity has several levels of complexity because antigenicity is defined by a host response, which is in itself one of the most heterogeneous biological phenomena known. Immune responses to antigens were once simply classified as humoral or cellular. Antibody classes and subclasses have since been extensively defined, and the antibody response to an antigen is now known to be very heterogeneous and complex. Cellular immunity was originally defined as that which could be adaptively transferred to normal animals with cells from syngeneic-immunized animals but not by passively transferring serum. Lymphocyte responses were later divided into B- and T-cell types and now a complex array of T-cell subclasses and intricate cellular networks to control both cellular and humoral responses have been proposed and partially elucidated. The appreciation of the importance of non-specific, or at least broad spectrum, killer mechanisms, such as activated macrophages, natural killer (NK) cells and natural antibodies (NAb), adds a further complexity, and these mechanisms may also be regulated by cellular control networks (19).

Antigens are substances which elicit an immune

response in an animal. The definition is thus arbitrary; host factors may influence the response immensely. If three animals are injected with a homogeneous tumor cell population and one animal responds by making antibodies, one by producing killer T-cells and one by not responding at all, the classification of the tumor cell as being antigenic or not would obviously depend upon the animal from which lymphoid cells or serum were collected and upon the method of assay (i.e., an assay for antibodies or an assay for killer T-cells). Furthermore, a cell surface determinant can be differentially able to induce an immune response (defined here as immunogenicity) and to be detected by humoral or cellular efferent mechanisms.

Clearly, the demonstration of antigenic heterogeneity within cancers, thought to be complex mixtures of clonal variants consequential to tumor progression, should not be unexpected.

Prehn (18) compared the immunogenicity of paired sublines derived from opposing poles of ten MCA-induced murine tumors. Pairs were described which differed in specificity and antigenic strength. Immunizing capacity (i.e., immunogenicity) did not necessarily correlate with susceptibility to the immune response (i.e., antigenicity). At about the same time as Prehn's work, the emergence of strongly antigenic variants of the weakly antigenic L1210 tumor following drug treatment of tumor-bearing mice was first described (20, 21, 22). Comparing parental L1210 and three sublines selected by resistance to methylglyoxal bis (guanylhydrazone), 4,4′-diacetyldiphenylurea bis (guanylhydrazone), or guanazole, Fuji and Mihich (23), found that, although the drug-resistant sublines were far more immunogenic/antigenic than the parent, the differences were due to quantitative expression of a common tumor-associated antigen. The L1210 guanazole-resistant subline was far more immunogenic and antigenic than the other cell lines as assessed by a plaque forming cell (PFC) assay in syngeneic DBA/2J mice. The common antigen could elicit both non-T-cell-mediated cytolysis in vitro detectable with the PFC assay (24) and transplantation resistance in vivo (25). Monoclonal antibodies which detected an antigen common to the L1210 sublines also reacted with spontaneous C3H mouse mammary tumors (26, 27) suggesting that the common L1210 antigen is the mammary-leukemia-(ML) antigen (28).

Other investigators have been able to demonstrate L1210 sublines which express antigens with different specificities. Antigenic variants may be induced (29), but variants also exist in the parental population (30). Killion (30) isolated L1210 subpopulations by lectin-nylon chromatography. Concanavalin A (Con-A), fucose-binding protein, ricin-communis agglutinin or phytohemagglutinin (PHA) bound to nylon fibers were used to bind different L1210 subpopulations. Subpopulations were then eluted with carbohydrate competitors of the lectins. Nicolin et al. (29) reported that treatment of mice bearing four different L1210 clones with 5-(3, 3 dimethyl-1-triazeno) imidazole-4-carboxamide (DTIC) induced the expression of new antigens on the DTIC/clones not found in the parental clones. The DTIC-variants of the clones shared a common antigen but also expressed DTIC/clone-specific antigens.

Miller and Heppner (31) described the immunogenic and antigenic activity and cross-reactivity of five tumor subpopulations derived from a mammary tumor which arose in a BALB/cfC3H mouse. Utilizing two in vitro assays and a Winn assay in vivo, we found that at least two determinants were involved and the expression varied markedly between tumor subpopulations. One antigen was considered to be a murine mammary tumor virus (MuMTV) antigen and one a unique antigen. Three of the subpopulations possessed both antigens: one expressed only the viral antigen, and one expressed only the unique antigen. Expression of the antigens varied both qualitatively and quantitatively; A determinant could be expressed so that a subpopulation could induce immunity detectable in the assays but not be sensitive to that immunity or vice versa.

A murine MCA-induced fibrosarcoma was found to exhibit a similar but even more complex system of determinants (32). Cloned subpopulations of the tumor were obtained by dilution cloning after two subcutaneous (s.c.) passages. From transplantation immunity studies with three clones (clones 10, 27 and 34) and the parental fibrosarcoma, Wang et al.

(32) proposed a model involving four determinants expressed differentially on the three clones. One antigen was shared by clones 10 and 27, one shared by clones 10 and 34, one unique to clone 27 and one unique to clone 34. Thus, although some common antigens were expressed by multiple populations, no antigen was shared by all cells. The dose of MCA used to induce this tumor, 0.5 mg MCA, is generally regarded as a low dose, but the origin of the tumor may well have been multi-focal (33) which could be the source of much of the antigenic heterogeneity seen in this tumor as well as in other MCA-induced tumors found to be antigenically heterogeneous (34–42). However, a low dose of MCA (5 μg) was recently reported to induce tumors of a single-cell origin (43).

Pfreundschuh and Cravioto (44) determined that a methyl-nitrosourea-induced rat glioma elicited antibody production in syngeneic rats. Comparison of the parental tumor and a clone revealed two glioma-specific antigens: One found only on the clone, and one found on both the parental tumor and the clone.

Urban et al. (45) isolated five progressor lines from the UV-induced fibrosarcoma 1591. Tumor 1591 induces a strong, specific T-cell-mediated immunity in C3H mice and usually regresses after temporary growth in normal mice. All five progressor lines had lost the TSTA of the parental tumor. The parental 1591 and four of the five progressor lines expressed an antigen detected by a hybridoma antibody.

Intratumor heterogeneity has also been described for the guinea pig line 10 hepatocarcinoma (46), human mammary tumors (47, 48), human osteogenic sarcoma (49) and human gliomas (50).

Immunologic heterogeneity of metastases

The realization that subpopulations with different antigenic profiles exist within primary tumors has spawned the popular hypothesis that metastatic subpopulations may represent the less antigenic cells of the primary tumor. However, comparisons of antigenic properties of cells from primary tumors and those from metastases have generally shown that metastatic cells may express the same or different antigens than primary cells and may or may not be less antigenic. The main conclusion one can draw regarding the antigenic properties of metastatic cells is that metastatic subpopulations are not unique but reflect the heterogeneity found in the primary tumor.

Kim (51) induced mammary tumors in immunosuppressed rats by oral administration of MCA. Tumor subpopulations were not tested, but three individual metastasizing tumors were unable to induce transplantation resistance in syngeneic rats whereas two individual non-metastasizing tumors did induce resistance to rechallenge.

In some cases, cells from metastases have been found to be antigenic but not cross-reactive with cells from primary tumors (52–56). Two lung metastases (M0 and M1) from a single Fischer rat bearing a benzo[a]pyrene-induced fibrosarcoma (P) were compared in transplantation resistance tests by Sugarbaker et al. (52) M0, M1 and P all immunized syngeneic rats to subsequent challenge with the tumor used for immunization. M0 and M1 immunized animals to P and weakly to each other (i.e., latency was increased but no change in ultimate incidence). P did not immunize mice against either M0 or M1. Thus, the metastatic sublines and the parental tumor displayed one-way cross-reactivity (see below). A determinant which was antigenic but not immunogenic on P was both antigenic and immunogenic on M0 and M1. It should be noted that M0 and M1 were no more metastatic than P. The authors' hypothesis that concomitant immunity (CI) to the primary tumor exists at the time of metastasis and immunoselection results in metastases with altered TSTA would predict that the metastases from animals bearing M0 or M1 in intramuscular (i.m.) transplantation sites would again be antigenically distinct. This was not tested. Additional experiments demonstrated CI to P by spleen cells from P-bearers in Winn assays (52). CI to M0 or M1 was not tested and may have also been present. The investigators assumed that metastasis could not have occurred in the face of CI to the metastatic cells. This might be true, but its demonstration would require an assay for CI involving inhibition of metastasis.

A very similar series of experiments, utilizing an MCA-induced mouse fibrosarcoma, showed essentially the same thing (53). Seven of 18 lung metastases were selected based on having similar growth rates to the primary tumor. Metastatic variants (M#) which grew faster or slower than the parental (P) were not studied. This procedure probably minimized the antigenic heterogeneity detected in the subsequent experiments. Even so, a broad range of antigenic heterogeneity was detected for the seven metastatic lines studied, displaying both reciprocal and one-way cross-reactions with the parental line and the presence or absence of the ability to immunize against itself. M2 immunized against itself and P, but P did not immunize against M2 challenge. M6 was not immunogenic but was antigenic since animals immunized to P (but not to M6) were resistant to M6. M9 immunized against itself but cross-reactivity with P was not seen. Neither M10 nor M15 could immunize against either themselves or against P, and P did not immunize against them. Both M13 and M16 immunized against themselves and against P, and P immunized against both M13 and M16; 'no discernible change in tumor antigenicity had occurred in these two metastases.' The authors suggested that M13 and M16 had originated from late metastases when the primary was large, C1 weak or absent, and immunoselection ineffective.

Faraci (35) compared a primary MCA-mouse fibrosarcoma (MCA-P) and two metastatic variants (MCA-M1 and MCA-M2). Immunization with any of the tumors rendered the animals strongly resistant to all three tumors. Not satisfied with this demonstration of reciprocal cross-reactivities in vivo, an in vitro microcytotoxicity assay was used to demonstrate one-way cross-reactivity between MCA-M2 and the other two tumors. MCA-M2 cells were less sensitive to lymphoid cells from animals immunized to any of the three tumors, but lymphoid cells from MCA-M2 immune animals only were cytotoxic for MCA-M2 cells, not for MCA-P or MCA-M1 cells. The author interpreted these data derived in vitro as an indication of the development of a neoantigen on MCA-M2 which 'produces no immunity against the primary tumor or another metastasis.' Others might prefer to accept the data derived in vivo which clearly showed that MCA-M2 immunized mice were resistant to MCA-P and MCA-M1.

Proctor et al. (57) isolated cells from two metastatic nodules in the lung and one metastatic nodule in a lymph node as well as from a primary fibrosarcoma induced with benzo[a]pyrene in a rat. Immunization with any of the four significantly protected rats against challenge with any of the four tumor sublines injected intravenously. Similarly, cells isolated from a local subcutaneous (s.c.) nodule of the highly metastatic Lewis lung carcinoma (L-3LL) were reciprocally cross-reactive with M-3LL cells isolated from a tumor growing s.c. as a result of the transplantation of cells from a metastatic lung nodule of the Lewis lung carcinoma (58).

Pimm et al. (39) established paired sublines from eight different primary MCA-induced rat sarcomas by transplanting cells derived from opposing poles of the autochthonous tumors a la Prehn (18). The antigenic relationships between the pairs were determined by their abilities to induce transplantation resistance. Three of the pairs were not cross-reactive, four pairs were reciprocally cross-reactive, and one pair was not demonstrably immunogenic. In addition, three sublines were derived from metastases found in the rat from which one pair of primary sublines was established. The pair of sublines from that primary were not antigenically different. A subline from a peritoneal metastasis was antigenically indistinguishable from the pair of primary sublines. A subline from a lung metastasis and a subline from a kidney metastasis were not cross-reactive with the primary or peritoneal metastasis but were reciprocally cross-reactive with each other.

Cells derived from six individual metastases from a human patient with malignant melanoma were heterogeneous for several antigens (59). Of the six, one had a large amount of a tumor antigen detectable with the patient's serum, two had lesser quantities of that antigen, and the antigen was not detectable on three. The six metastatic sublines also differed in HLA-DR antigen expression and displayed quantitative differences with a battery of 17 antisera against a range of melanoma differentiation antigens.

Mantovani et al. (60) compared cells from a primary benzo[a]pyrene-induced mouse sarcoma, mFS6, with cell lines derived from nine individual lung metastases of mFS6. Seven of the metastasis-derived sublines were reciprocally cross-reactive with the parental sarcoma by transplantation resistance tests. Two sublines were not demonstrably immunogenic or antigenic; They did not immunize to self or to parental challenge and immunization with the parental line did not make animals resistant to these two sublines. The authors also determined the metastatic potential of the metastasis-derived sublines. After either intravenous (i.v.) or s.c. injection, some sublines were more metastatic and some were less metastatic than the parental mFS6. Alterations in antigenicity did not correlate with alterations in metastatic behavior. Thus, one non-antigenic subline behaved as the parent tumor after both i.v. and s.c. transplantation, and one was less metastatic than mFS6 after s.c. transplantation.

Collectively, these investigations illustrate that metastasis-derived tumor variants may or may not behave immunologically like the corresponding primary tumor but are not frequently less antigenic than primary tumors. Consider, too, that a primary tumor is a collection of subpopulations which may include subpopulations identical to the metastasis-derived variants and cross-reactivity could be demonstrable even though only a single subpopulation expressed that antigen.

Immunologic heterogeneity of preneoplastic cells

At some stage, when preneoplastic lesions precede malignant lesions, tumors contain mixtures of preneoplastic and neoplastic populations as well as normal cells which in some cases may be requisite for tumor growth (61). It is not clear if all cells in preneoplastic lesions are at equal risk to progress to malignancy or whether a minor population at high risk exists, and the antigenic relationship between the preneoplastic and malignant cells has not been well established. Lappé (62) hypothesized that the immune system maintains preneoplastic cells at a differentiated, non-malignant level by controlling cell surface component expression. When control breaks down, antigenic diversity within tumors is generated as a response to the selective pressure of that immune control. However, the data presented to support that hypothesis were meager.

Boone et al. (63) used C3H/10T1/2 cells as a model for preneoplasia. These cells produce tumors if implanted subcutaneously attached to plastic. From a population of parental preneoplastic cells, they describe three tumors so derived with non-cross-reactive, tumor-associated transplantation antigens; these antigens were not shared by the parental C3H/10T1/2 cells. An interesting finding was that two of the tumor lines arose from a population of cells which constituted only 1.3% of the original 10T1/2 cell line, as determined by detection of marker chromosomes, and yet the two had distinct antigens as demonstrated by transplantplantation resistance. These results are compatible with Lappé's hypothesis but other, more conventional, models of preneoplasia have not supported this hypothesis.

Lappé (64) found that an MCA-induced papilloma (premalignant) immunized syngeneic BALB/c mice to challenge with either the papilloma or a carcinoma which subsequently arose from the papilloma. Immunization with the carcinoma rendered mice resistant to challenge with either the premalignant papilloma or the carcinoma. Specificity was not demonstrated with cross-protection experiments with other tumors, however, so the common antigen might not have been unique TSTA.

Mouse mammary hyperplastic alveolar nodules (HAN) and ductal hyperplasias (DH) are well-characterized models for preneoplasia. Medina has described several transplantable HAN lines (65). Subpopulations of the tumors arising from the transplantable lines have not been tested, but individual tumors arising from a single HAN line may be analogous to tumor subpopulations. The antigenic relationships between HANs and related tumors and between tumors arising from common parental HAN lines has been described. Tumors arising from a HAN line, but in individual mice bearing transplanted HAN tissue, are cross-reactive in transplantation protection assays (66, 67) and in a microcytotoxicity assay in vitro (68). The detected antigen is tumor specific in that cross-reactions

between tumors arising from different HAN lines are not seen (66–68). The antigenic relationship between HAN and subsequent malignant tissue is not clear. Slemmer (67) reported that preneoplastic mouse mammary lesions expressed specific antigens which persisted unchanged in subsequent malignant tumors. Heppner et al. (68) were unable to detect cross-reactivity between D1 HAN and tumors derived from D1 lesions or between D2 HAN and tumors derived from D2 lesions. Recent results with the C4 HAN and resultant C4 tumors suggest that tumor and HAN share an antigen detectable in vitro, but immune effector cells are induced only by C4 tumor cells, not by C4 HAN cells (Miller and Heppner, unpublished).

Mechanisms of intratumor heterogeneity

Although the basis of intratumor immunologic heterogeneity may reside in qualitative differences among subpopulations in antigen content, quantitative differences in tumor-associated antigens, differences in antigenic topology and differences in other membrane properties undoubtedly introduce complex subtleties into the phenomenon. The result can be seemingly aberrant patterns of cross-reactivity between tumor subpopulations. Such patterns point out some of the difficulties in establishing 'tumor specificity'.

Miller and Blazkovec (69) used an in vitro chromium-release test to assess immune responses to two strain 13 guinea pig MCA-induced sarcomas (MCA-1 and MCA-2). MCA-1 cells were lysed by effector cells from guinea pigs immune to MCA-1 and not from guinea pigs immune to MCA-2, but the MCA-2 cells were lysed by either effector cell. It was suggested that the one-way cross-reactivity was due to a common determinant which was expressed properly on both cell lines to be a target for CTLs (i.e., it was antigenic) but was not immunogenic in vivo as expressed on MCA-2 cells. Miller and Heppner (31) described one-way cross-reactions between subpopulations of a single mouse mammary tumor with two in vitro assays for cell-mediated immunity (CMI) and the Winn assay. Miller et al. (70) found that one of those sublines, 410, immunized syngeneic mice against both sublines 410 and 168 in transplantation resistance tests; subline 168 did not immunize mice to either subline 410 or 168.

One-way cross-reactive relationships may appear after serial transplantation (71). Benzo[a]pyrene-induced mouse sarcomas lost their ability to immunize mice but were still rejected by mice immunized with earlier generations of the same tumor. The antithesis has been described for a mouse teratocarcinoma (72–74) in which antigenic sublines, isolated after treating the parental line with the mutagen N-methyl-N'-nitroso guanidine, immunized syngeneic mice to themselves and to the parental teratocarcinoma which itself was not immunogenic. Vaage (75) characterized the growth and immunology of C3H mammary tumors during serial transplantation and found that three tumors lost immunosensitivity while retaining immunogenicity. Prehn (18) has also reported that the immunizing capacity and susceptibility to the immune response did not correlate for subpopulations of MCA-induced tumors.

Variability in the expression of a cell surface determinant is a fundamental type of immunologic heterogeneity. The frequency with which one-way cross-reactions have been described for tumor subpopulations (18, 23, 31, 35, 52, 70, 71, 74, 75) probably reflects this variability.

Several mechanisms can be envisioned by which one-way cross-reactions could occur. Quantitative differences in determinant expression might be important. Expression of immunogenicity might require lower concentrations of antigen than expression of immunosensitivity or vice versa. Fenyo et al. (76) selected an immunoresistant line of the strain A Moloney lymphoma, YAC. The resistant subline, YAC-IR, was obtained by injecting YAC cells into syngeneic animals preimmunized with irradiated YAC cells. The selected cells were resistant to C′-dependent lysis with anti-YAC sera but not with anti-$H2^a$ sera. Immunofluorescence assays indicated that the same proportion of YAC and YAC-IR cells express Moloney virus antigens (80–90%), but the positive YAC-IR cells were found to express ten-fold less antigen by quantitative absorption tests. Both YAC and YAC-IR

lines were, however, equally immunogenic in inducing humoral responses and cellular responses.

Quantitative expression of cell surface antigen may be cell-cycle dependent, but different antigens are expressed maximally during different stages (77–83). Rauscher murine leukemia virus and Kirsten murine sarcoma-leukemia virus antigens were expressed during G2 and S but not in M or G1 (81). The expression of Moloney leukemia virus antigen on YCAB lymphoma cells during the cell cycle was described in a series of papers (77, 78, 80). The antigen was present throughout the cycle and was expressed in a form which bound anti-Moloney leukemia virus antibodies (Ab) and activated complement (C′). However, the YCAB cells were lysed by the Ab-C′ reaction only during G1 (80). A quantitative absorption protocol demonstrated that G1 cells had 2–3 times as much antigen as S-phase cells (77). Thus, the YCAB cells were most sensitive to Ab-C′ lysis when the most antigen was expressed.

However, decreased susceptibility to efferent immunity can not always be explained by quantitative changes. A human cell line maximally expressed HLA antigens during G2 and S, but the ability of Ab and C′ to lyse the cells did not correlate with antigen content of the cells (79). Cells of the guinea pig line 10 and line 1 hepatomas have comparable concentrations of Forssman antigen (determined by C1- -fixing sites), but the line 1 cells were much more readily lysed in the presence of anti-Forssman antiserum and C′ (84). Line 10 cells were readily lysed by a specific anti-line 10 serum in the presence of C′. Thus, the resistance of line 10 cells to lysis was not a general property but was antigen-dependent. The anti-Forssman antiserum was an IgM preparation so the activation of C′ did not, presumably, require any particular array of the cell-surface antigen.

The length of time a determinant is expressed on the cell surface might dictate whether it is immunogeneic and/or antigenic. Thus, immunologic variations might occur with altered growth kinetics of cell subpopulations and might be partially responsible for the relative immunologic privilege, which is tissue dependent, at various anatomical sites (61, 85–91).

Immunogenic tumor cells can escape efferent immune destruction via antibody-induced antigenic modulation (92, 93). Stackpole (94) reported that IgG but not IgM induced modulation of thymus-leukemia antigens in mice. Imagine two subpopulations: subpopulation A which induces an IgM response and population B which induces an IgG response. Anti-A sera would lyse both populations but anti-B serum would not. Thus, although both subpopulations induced antibody formation, the data would indicate that A was both immunogenic and immunosensitive, but B was immunosensitive but not immunogenic, i.e., a one-way cross-reaction. Shedding of cell surface antigens could also result in apparent one-way cross-reactions. Clones of an MDAY-D2 tumor differed in tumorigenicity (95), but no antigenic differences between the parental, the tumorigenic clones and poorly tumorigenic clones were detected serologically. However, the poorly tumorigenic variants did not shed cell-surface components, whereas the parental MDAY-D2 and the tumorigenic variants were reported to be prolific shedders. The implication was that the non-shedding variants were highly susceptible to efferent transplantation immunity.

Sialic acid can mask the presence of antigens on tumor cell surfaces. Shearer et al. (96) selected variant subpopulations of L cells which were resistant to C′-dependent lysis by antiserum able to lyse the unselected L cells. The apparent loss of antigenicity by one variant was due to antigenic masking by sialic acid. Neuraminidase treatment rendered them sensitive to the Ab-C′ reaction.

Viral antigens and tumor-specific antigens on murine mammary tumor virus (MuMTV)-induced tumors are differentially masked by sialic acid (97). Neuraminidase treatment increased the ability of the mouse mammary tumors to specifically immunize mice but did not facilitate cross-protective immunization between tumors, even in MuMTV-free mice. Intratumor injections of spontaneous MuMTV-induced mammary tumors with neuraminidase induced regression, but new tumors could simultaneoulsy arise in other mammary glands (98).

Tumor subpopulations may differ quantitatively in H-2 expression (33, 76, 99), may have altered H-2 determinants (100) or may express inappropriate H-2 antigens (101, 102). A requirement for

responding/effector cells to share major histocompatibility antigens with stimulator/target cells has been described for responses to cell surface virus antigens (103, 104), including tumor virus antigens (105, 106) and other tumor-associated surface antigens (107, 108). H-2 restriction is another possible explanation for one-way cross-reactions. BZ-L2C, one of five sublines of a strain 2 guinea pig leukemia described by Forni et al. (109), was not immunogenic. All of the other four sublines immunize strain 2 guinea pigs to any of the sublines, including BZ-L2C. The BZ-L2C variant was also unique among the five sublines in its lack of Ia antigen, and it was suggested that the presence of Ia antigen was necessary to elicit a response to the L2C tumor-associated transplantation antigen. I-region compatibility is apparently not necessary for efferent expression of immunity (110, 111).

H-2 restriction is not always absolute and may be more restrictive for some tumor lines than others (112). In chromium release tests (CRT), H-2b splenocytes, stimulated in vitro with trinitrophenyl (TNP)-modified H-2b splenocytes, were cytotoxic for TNP-modified H-2b tumors and, to a lesser extent, for modified H-2k tumors but were not lytic for modified H-2d tumors. Similarly, H-2k splenocytes, stimulated by modified H-2k splenocytes, were cytotoxic for modified H-2k and H-2b but not H-2d tumors. H-2d splenocytes, stimulated with modified H-2d splenocytes, were cytolytic for TNP-modified H-2k and H2b as well as to modified H-2d tumors. Two tumor subpopulations might express the same tumor-associated antigen on the cell surface but differ immunologically due to loss of H-2 or because one of them expresses an inappropriate H-2.

Expression of inappropriate H-2 antigens might allow the 'escape' of a tumor subpopulation via H-2 restriction. However, inappropriate H-2 antigens could also make a tumor subpopulation more susceptible to another host resistance phenomenon, allogeneic inhibition (113, 114).

Minor alterations of antigens have been detected by agents which bind specifically to carbohydrate side chains such as the Semliki Forest Virus (100) and various lectins (30, 115–118). L1210 cells were isolated by affinity chromatography with Con-A, and, depending upon the sugar used to elute, various subpopulations of Con-A bound L1210 cells were more or less successful as tumor vaccines in combination with chemotherapy (116).

Receptors for natural killer (NK) cells are also expressed heterogeneously within tumors. Differences in sensitivity to NK-killing have been described for tumorigenic and non-tumorigenic variants of L cells (119), variants of YAC-1 cells (120), for clones from the SL2 lymphoma of DBA-2 mice (121), from the Lewis lung carcinoma (122), and for progressor lines of the UV-induced fibrosarcoma 1591 (45). Often regarded as being non-specific, Takasugi et al. (123) used cross-competition cytolytic assays to demonstrate that 'what appeared to be non-selective effects of natural cytotoxicity were in fact highly specific. Each effector suspension included natural effector cells that recognized and reacted specifically with many different antigens on target cells, resulting in overall non-selectivity.' NK-receptor molecules may be extracted from NK-sensitive tumor lines but not from NK-resistant tumor lines and a 240K dalton molecular weight fraction specifically inhibited NK-mediated lysis of the NK-sensitive cell line from which it was prepared (124). Supernatants from cultured tumor cells contain glycoproteins which inhibit NK-mediated lysis in a CRT (125), and the inhibition depends both on the target and the effector cell source. The NK-receptor molecules may be differentiation antigens. Inducement of differentiation with agents such as sodium butyrate, dimethylsulfoxide or 12-tetradecanoylphorbol-13-acetate may alter the NK-sensitivity of tumor cells (126–128).

Macrophage-resistant tumor cell lines are rare (17, 119, 129–132), but Berd and Mastrangelo (133) reported the isolation of an L1210 line which was resistant to killing by *Corynebacterium parvum* (CP)-activated peritoneal exudate cells (PECs). Evidence that the effector PECs were macrophages included adherence, phagocytic activity, sensitivity to silica poisoning, absence of cytolytic activity in splenocytes from CP-treated mice and equivalent activity of PECs from both young and old CP-treated mice. Nicolson et al. (134) reported that variants of the RAW117 lymphosarcoma which differed in metastatic properties and viral antigen

expression also differed in sensitivity to macrophages in vivo. Heterogeneity in sensitivity to killing by activated macrophages is, however, apparently very limited.

Variants differing in susceptibility to C′-dependent NAb lysis have been isolated by cloning from L5178Y lymphoma (135) and SL2 lymphomas (129). NAb lysis may involve synergistic cooperation between antibodies against multiple specificities on a tumor cell. Colnaghi et al. (136) obtained four monoclonal NAbs by hybridization splenocytes from normal mice selected for high NAb activity to EL4 cells. The antigens detected by the four monoclonal NAbs had different specificities: Two were virus-related, one was present on EL4 cells and on normal fibroblasts and one was present on EL4 cells and embryonic cells. The latter antigen was also thought to be cell-cycle dependent. Tumor cells, possibly through derepression, express many antigens which may be present on normal cells of other tissue types and on cells at various levels of differentiation. Colnaghi et al. (136) suggested that 'the higher number of antibody molecules that react with tumor cells compared with normal cells may render the former susceptible to the C′-dependent lysis, to which the latter are resistant due to the low epitope density on their cell surface.' Synergism in C′-dependent lysis of human melanoma cells has been described for two monoclonal antibodies directed towards two determinants of an individual antigen (137).

Antagonistic interactions also occur. Both effector and suppressor cells are generated by both tumor specific transplantation antigens and common tumor associated antigens (138). T-suppressors elicited by even weak tumor-associated antigens could inhibit induction of immunity to strong tumor specific transplantation antigens.

Subpopulation interactions in intratumor immunologic heterogeneity

Most of the data on immunologic heterogeneity of tumors has been obtained by comparing immune parameters of isolated tumor cell subpopulations. The subpopulations do not exist in isolation. Interactions occur in mixed populations which alter phenotypic expression of growth rates (70) and drug sensitivities (139).

When subpopulations co-exist, immunologic parameters may also be altered by the occurrence of interactions. The results of Nowotny and Grohsman (140) suggested that the relative proportion of two subpopulations of the TA3 mouse mammary adenocarcinoma, which display one-way cross-reactivity, might dictate whether immune rejection of immunoresistant cells or the escape of immunosensitive cells will occur. Mixtures of the two subpopulations injected into normal mice resulted in either the growth of both lines when the immunoresistant line was in excess, or the rejection of both when the immunosensitive line was in excess.

Olsson and Ebbesen (141) identified four antigenic subpopulations from a spontaneous AKR lymphoma which they inexplicably called 'clones' A, B, C and D. Interpretation of their results are difficult because an immunizing mixture of all four 'clones' were not pre-irradiated whereas 'clones' were pre-irradiated before use as single agents or as combinations of two or three. However, treatment of leukemia-bearing mice with a mixture of all four 'clones' was much more efficient (92% cures) than treatment with A (32% cures), B (0%) C (0%), D (0%), A + B (33%) or A + B + C (45%).

Viral infection of tumor cells can result in the immune rejection of non-infected, but otherwise, identical, tumor cells. Al-Ghazzouli et al. (142) described a BALB/c fibrosarcoma, which was free of detectable endogenous C-type viruses, highly tumorigenic and weakly immunogenic. Infection with Rauscher murine leukemia virus made the fibrosarcoma highly antigenic, capable of inducing an immune response which rejected both the infected and non-infected forms.

We have described the antigenic heterogeneity of five tumor cell subpopulations (66, 67, 68H, 168 and 410) derived from a single BALB/cfC3H mouse mammary tumor (31). Experiments in which mice were immunized with mixtures of two sublines derived from a single mouse mammary tumor indicated that the specificities of the responses to the mixtures were not simply the sum of the responses to the individual subline (143). Alone in

BALB/cfC3H mice, tumor 410 induces immunity to both itself and tumor 168, whereas tumor 168 does not induce resistance to transplantation of either 410 or 168. Immunization with a mixture of 168 and 410 leads to resistance to transplantation of either 410 or 168 (70). Thus, the immune response to this mixture reflects the sum of the responses induced by the individual subpopulations and no immunologic interaction is indicated. We have also found that lymph node cells from BALB/c mice immunized with either tumor 66 or tumor 67 inhibit the growth of tumor 67 but not 66 in Winn assays (143). However, lymph node cells from BALB/c mice immunized with a mixture of 66 and 67 inhibit the growth of 66 but not 67. The immune response to this mixture indicates the simultaneous appearance of a new reactivity (against 66) and loss of reactivity (against 67). Thus, with various pairs of the subpopulations, all possible events may occur after immunization. These experiments demonstrate that the host response to any subpopulation of a tumor is modulated by other subpopulations so that the response to a complex heterogeneous tumor can not be predicted by the responses to individual subpopulations.

It is not known what proportion of an autochthonous tumor a subpopulation represents nor how many different subpopulations were present in that original tumor. Indeed, the existence of any subpopulation in the original tumor is never assured and heterogeneity may be generated as subpopulations are isolated (95, 144, 145). 'Real' tumors undoubtedly induce host responses which can not be reproduced by tumor subpopulations, mixtures of subpopulations or even by tumors produced by transplanting pieces of primary, spontaneous tumors. The dynamics of progression, with continuously evolving subpopulations, initiate complex immunologic cascades which develop in an immunological environment which has resulted from all previous responses.

The perplexities which intratumor immunologic heterogeneity imparts to immunotherapy is evident. Add the contingencies of subpopulation interactions and the weak immunogenicity of spontaneous tumors and the prospects for specific immunotherapy seem dim. Stimulation of nonspecific resistance to tumors is an alternative approach. Fidler and colleagues have demonstrated the feasibility of such an approach by systemically activating macrophages in vivo by injecting liposomes containing macrophage-activating factor or muramyl dipeptide to induce regression of metastases (146–148). It may, however, also be possible to exploit some of the phenomena involved in immunologic heterogeneity to develop new approaches for specific immunotherapy. Active immunotherapy might be beneficial against even non-immunogenic cells if one-way cross-reactivities and subpopulation interactions could be created and/or selectively enhanced. This might involve the isolation of an active, 'controlling' subpopulation or the creation of a controlling subpopulation by treatment with mutagens, by manipulation of major histocompatibility antigen expression, by alteration of subpopulation cell cycles, by infection with a virus or by chemical modification. The great diversity of antigenic expression on tumor cells may eventually be the means by which passive immunotherapy succeeds. The synergistic effects of a large panel of monoclonal antibodies, reminiscent of multivalent antisera previously used in some infectious diseases, directed against determinants which occur only infrequently on normal cells may be used to preferentially kill tumor cells.

An understanding of the mechanisms by which heterogeneity is created and maintained in a cancer might alter combination therapies also. A treatment which selectively kills a 'controlling' subpopulation might ultimately be detrimental to the host. Even though an initial regression might occur, after the sensitive 'controllers' were gone, the subsequent growth of the remaining cancer might far exceed that of the original.

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