ROLE OF CELL SURFACE RECEPTORS IN THE REGULATION OF NON ADAPTIVE IMMUNE RESPONSE BY THE LARGE GRANULAR LYMPHOCYTES

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Summary. - *The 65 kDa Fc receptor (FcR) molecule for human IgG recognized by the MoAb AB8.28 is coexpressed on the large granular lymphocytes with the 55 kDa 3G8 FcR molecule. The AB8.28 molecule has a unique distribution among T and B cell leukemias and non-lymphoid tissues. Biochemical comparisons revealed structural identity of the AB8.28 molecule expressed on different lymphoid and nonlymphoid tissues. AB8.28 and 3G8 molecules are low avidity FcR which are structurally similar but not identical, as demonstrated by chromotryptic peptide mapping, electrophoretic mobility and tissue distribution. The AB8.28 molecule has been successfully used to transduce signals able to trigger NK cells, which upon activation release the content of granul with local toxic activity. These data collectively indicate that AB8.28 MoAb identifies a new class of IgG FcRs with relevant biological properties on NK cells.*

Riassunto (Ruolo dei recettori della superficie cellulare nella regolazione della risposta immunitaria non adattativa indotta dai linfociti granulati grandi). - *La molecola recettoriale (FcR) della porzione Fc dell'IgG umana avente 65 kDa che viene ricoperta dall’anticorpo monoclonale (MoAb) AB8.28 è coespressa sui linfociti granulati grandi insieme alla molecola 3G8 FcR di 55 kDa. La molecola AB8.28 ha una distribuzione particolare tra le cellule leucemiche di tipo T e B e tra le cellule di tessuti non linfoidi. Le comparazioni biochimiche hanno rivelato identità strutturali della molecola AB8.28 espressa su differenti tessuti linfoidi e non. Le molecole AB8.28 e 3G8 sono molecole FcR con bassa avidità e sono strutturalmente simili ma non identiche come dimostrato dalle mappe peptidiche derivate dalla digestione chimiotritica, delle mobilità elettroforetiche e della loro distribuzione nei tessuti. La molecola AB8.28 è stata usata con successo per indurre segnali capaci di scatenare le cellule NK, le quali una volta attivate rilasciano il loro contenuto formati da granuli con attività tossica a livello locale. Quest’insieme di dati indica che il MoAb AB8.28 identifica una nuova classe di recettori FcR dell’IgG aventi importanti proprietà biologiche nelle cellule NK.*

Introduction

During the last years, the monoclonal antibodies (MoAbs) have been extensively used to characterize cell surface molecules which exert relevant biological function for the life of the individual cell and for its relationship with the other cells. After the use of MoAbs as a tool to purify, characterize and compare such structures, it gradually appeared that in many cases where the target molecule displayed receptorial activity - the binding of the specific antibody is able to trigger a signal similar to that brought by the natural ligand. In a broad way, agonistic antibodies are able in selected instances to mimic membrane signals mediated either by soluble factors or signals intervening after cell-cell interactions. The transmission of transmembrane signals via antagonistic antibodies has many advantages, including unexpensiveness and easiness whereas the major drawback is represented by the fact that they only mimic the physiological signal in an extent which is not fully known and which cannot be taken as representative of the natural picture. The specular side of the story is represented by the several reagents which exert antagonistic effects, e.g. anti-HLA class I or II MoAbs [1, 2]; in general, the inhibition of a selected function is a finding often seen with caution since the effects are observed at very high concentrations and in the majority of cases is not eliminated the possibility of effects mediated through the receptors of the Fc region (FcR) of the immunoglobulin G (IgG).

The agonistic MoAbs have been classified as transducers of competence signals (when the MoAb is only able to activate cells) and of progression signals (when they act only in synergy with companion stimuli). The effects of agonistic MoAbs to human T and B cells differentiation antigens are depicted in Table 1. More details can be found in ref. 3.

Our interest is mainly addressed to the use of complement receptors recognized by the MoAb CB04 [4] and to the analysis of FcR expressed on natural killer cells either in structural or functional terms. The present paper only deals with anti-FcR reagents, which identify a
Table 1. - Surface molecules transducing signals in T and B lymphocytes

<table>
<thead>
<tr>
<th>Molecule</th>
<th>T cells</th>
<th>B cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3, Tp19-29</td>
<td>competence signal which needs accessory cells</td>
<td>CD20, Bp35</td>
</tr>
<tr>
<td>CD2, Tp50</td>
<td>epitope-dependent activation</td>
<td>CD21, Bp140</td>
</tr>
<tr>
<td>CD5, Tp67</td>
<td>increased production of IL-2, progression signal</td>
<td>Bp50</td>
</tr>
<tr>
<td>Tp44</td>
<td>increased production of IL-2 and IL-2R, progression signal</td>
<td>CD22, Bp135</td>
</tr>
<tr>
<td>Lp220</td>
<td>increased IL-2R-positive cells, progression</td>
<td>CD23, Bp45</td>
</tr>
</tbody>
</table>

structure whose natural ligand is known. The receptor molecule that selectively binds the Fe domain of human IgG on the surface of hemopoietic cells has first been described functionally and subsequently by means of polyclonal and MoAbs [5, 6]. These studies have revealed that there is a family of FcR represented by at least three groups of molecules, that have been classified according to several criteria, including molecular mass, affinity to monomeric IgG and tissue distribution. So far the picture seems to include a high affinity receptor of 72 kDa, termed FcRI, expressed on monocytes [7], a low affinity receptor of 50-70 kDa, termed FcRlo, present on large granular lymphocytes (LGL), neutrophils and macrophages [8-10] and an additional low affinity receptor of 40 kDa, termed FcRII, found on platelets, eosinophils and some hemopoietic cell lines [11-14]. We have produced and characterized a MoAb AB8.28 which displayed functional features of FcR [15]. Taking into account several parameters (e.g. function, tissue distribution, structure, etc.), we confidently included it among the reagents defined as low avidity anti-FcR MoAbs, which includes the molecule recognized by several MoAbs such LeuL, B73.1, VEP13 and 3G8. All these reagents define the cluster differentiation 16 (CD16), following the nomenclature proposed by the 2nd International Workshop of Antigen Differentiation [16]. The strict comparison of AB8.28 MoAb with the companion reagents resulted unsatisfactorily, since many evidences were addressing to difference instead of coincidence, as evidenced by several lines of evidence, reported in the original description [15] and in successive reports [17, 18]. For these reasons, the original study on the AB8.28 molecule was extended by further investigations which demonstrated that reaction of LGL FcR with MoAb AB8.28 linked to an insoluble matrix induces cell activation (as evidenced by the expression of HLA class II antigens, interleukin-2 and transferrin receptors) and release of platelet activating factor (PAF) [19] and so far unknown factor(s) enhancing the formation of macrophage/granulocyte colonies (L. Matera, Turin, personal communication). Ontogenetic analysis of the AB8.28 molecule on hemopoietic cells has shown that fetal thymus cells are AB8.28-positive [20], while their adult counterpart and resting peripheral blood T cells are both negative [15]. However, T cells become clearly positive upon activation in mixed lymphocytes reaction (MLR) cultures [15], thus suggesting a possible linkage between the expression of the AB8.28 FcR and cell activation stage.

The AB8.28 molecule showed some conflicting features with those attributed to CD16 receptor. In fact, the epitope recognized by the AB8.28 was different from that of all the MoAbs included in the CD16 cluster and further comodulation experiments ruled out molecular identity between the determinants recognized by AB8.28 and CD16 MoAbs. These findings were strengthened by the results of the in vivo analysis of the expression of these molecules in selected malignancies, which confirmed the discrepancies [21].

We approached the open queries on the nature of the AB8.28 FcR by comparing the expression and molecular structure of this molecule with the FcR recognized by the MoAb 3G8 taken as prototype of the CD16 group. Summarizing in advance our findings, the results indicate that the AB8.28 and 3G8 FcRs are different in the tissue distribution, since the former is detectable on a variety of cells such as T and B leukemias, in vitro cultured fibroblast, sarcoma and carcinoma cell lines, while the 3G8 FcR was not present on any of these cells. Moreover our data clearly demonstrate that AB8.28 and 3G8 FcR molecules differ not only in their phenotypic distribution but also structurally, as evidenced through Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and chymotryptic peptide mapping. These data may provide a clue toward attributing to AB8.28 molecule a role as a fourth class of IgG FcR. All the data will be analyzed in detail in the next section.
Results

Two anti-FcR reagents were used in the present study to biochemically characterize the FcR molecules expressed on LGL and on other cell populations. MoAb AB8.28 (an IgG1 isotype) was developed by immunization of BALB/c mice with peripheral blood lymphocytes of a healthy donor [15]. MoAb 3G8 (IgG1) was also described elsewhere [9] and was generously supplied by Dr. J. Unkless (Mount Sinai School of Medicine, New York, N.Y.). The FcR's were analyzed on LGL and T cell preparations which were obtained as described previously [22, 23].

Moreover, we extended our analysis to several continuous cell lines growing in vitro. Our selection included T (HPB-ALL, MOLT4) and B (JOSH, JY) cell leukemias, foreskin and fetal lung fibroblasts (kindly supplied by Dr. M. Eisinger, Memorial Sloan-Kettering Institute for Cancer Research, New York, N. Y.), and a panel of tumor cell lines including rhabdomyosarcoma WRMS-186, liposarcoma WLS-160, testicular carcinoma Frt and breast carcinoma MT-29 [24]. Other details on the characteristics of these lines are referred in Ref. 25.

The usual approach in comparing two structures is the analysis of the immunoprecipitates in SDS-PAGE in appropriate conditions. For the purpose, we labeled 25-75 x 10^6 cells using the lactoperoxidase method with 5 mCi of Na^125I (Amersham International; 1 Ci = 37 GBq) and lysed in 2 ml of 0.15 M NaCl 0.01 M sodium phosphate, pH 8.3/0.5% Nonindet P-40/0.001 M phenylmethylsulfonl fluoride. Prior to immunoprecipitation, bovine hemoglobin was added to cell lysates at a final concentration of 1% to reduce nonspecific binding of labeled material to antibody-coated beads. Immunoprecipitation was carried out by incubation of the labeled cell lysates with antibody-coated goat anti-mouse IgG agarose beads (Sigma). The material was eluted from the beads with Laemmli sample buffer [26] for reducing (with 2% mercaptoethanol) SDS-PAGE or with isoelectrofocusing (IEF) sample buffer [27] for two-dimensional (2D) gel electrophoresis. The results are shown in Fig. 1, where MoAbs AB8.28 and 3G8 immunoprecipitated from the radioiodinated LGL cell lysates two molecules with a different molecular mass of 65 and 55 kDa, respectively. In contrast, peripheral blood T lymphocytes were negative for the 65 kDa AB8.28 molecule, and only reduced quantity of the 55 kDa molecule was precipitated by the 3G8 antibody (Fig. 1) which confirmed a previous report that only 6% of T lymphocytes express the 3G8 epitope as demonstrated by immunofluorescence and likely corresponding to the T subset [28]. Nevertheless, MRL-activated T blasts were clearly positive for the 65 kDa AB8.28 molecule [15], whereas ConA-activated T blasts remained negative (data not shown). The AB8.28 molecule, but not the 3G8, was also clearly demonstrable on the surface of HPB-ALL, MOLT4, JOSH and JY leukemia cell lines as well as in WRMS-186, WLS-160 sarcoma and MT-29.

Fig. 1. - SDS-PAGE of the FcR molecules immunoprecipitated with AB8.28 and 3G8 monoclonal antibodies from the radioiodinated peripheral blood LGL and rhabdomyosarcoma WRMS-186 cell lysates. C = controls.

Frt carcinoma cell lines as shown by SDS-PAGE, whereas 3G8 may not. Similarly, in vitro cultured foreskin and fetal lung fibroblasts expressed only the 65 kDa AB8.28 molecule.

To rule out the possibility that AB8.28 MoAb was reactive with similar epitope on different molecules, the target molecule of AB8.28 MoAb has been immunoprecipitated from lymphoid and nonlymphoid cells. The AB8.28 molecules immunoprecipitated from LGL, HPB-ALL, WRMS-186 and MRL-activated T cell lysates displayed identical patterns on IEF-SDS-PAGE 2D gels (Fig. 2). To further investigate the nature of these molecules and their composition in carbohydrates, we resuspended the immunoprecipitates in 50 mM Tris-HCl buffer, pH 7.8, containing 0.1% SDS, 1% NP-40 and 1% 2-mercaptoethanol, and boiled for 3 minutes. The cooled supernatant was then subjected to 5% (v/v) endo-F treatment for 8 h at 37 °C followed by SDS-PAGE. Details are referred in [29]. Treatment of AB8.28 precipitates from these cell lysates with endo-F resulted in replacement of the 65 kDa band by the 45 kDa band (not shown), thus suggesting that these AB8.28 molecules have similar N-linked carbohydrates.

Once shown the structure of the AB8.28 isolated from different tissues, we extended the analysis of this and 3G8 molecules at a peptide level.

SDS-PAGE was performed according to Laemmli [26] on vertical slab gels of 11% acrylamide and the 2D
Fig. 2. - 2D gel pattern of the AB8.28 FeR molecule immunoprecipitated from the radiiodinated rhabdomyosarcoma WRMS-186 cell lysate. Only segment of the autoradiograph is shown. As stated in the text, the patterns of AB8.28 molecules precipitated from different tissues cell lysates were identical; therefore only one was chosen for illustration.

The analysis was performed as described previously [29]. IEF slab gels for first-dimensional resolution of 2D analysis were constructed with pH 3.5-10 ampholines (LKB). The IEF slabs were then cut into strips for the second-dimension resolution by SDS-PAGE performed on vertical slab gels. Gels were dried on filter paper under vacuum and autoradiographed on Kodak X-Omat R film with a Dupont Cronex intensifying screen at -70 °C. The chymotryptic peptide mapping is a sophisticated way of comparing similar molecules and it permits to take peculiar features of the structures under investigation. The test was done according to Elder et al. [30], using radioiodinated and immunoprecipitated molecules isolated from slices of Laemmli slab gel and subjected to chymotrypsin digestion (50 μg/ml of enzyme in 1% ammonium bicarbonate, pH 8.3) for 18 hours at 37 °C. The comparison between the 65 kDa AB8.28 molecules isolated from the SDS-PAGE slabs (Fig. 1) and subjected to chymotryptic peptide mapping, has revealed no difference in the composition of tyrosine-containing peptides (Fig. 3 A and B). We also compared chymotryptic digests of the 65 kDa AB8.28 and the 55 kDa 3G8 molecules precipitated from the same LGL cell lysate. As illustrated in Fig. 3 (panels A, C and D), the analysis of the maps has shown that the peptide composition of the two molecules is clearly different. However, a careful analysis of these maps permitted to trace a few peptides common to both structures.

Fig. 3. - Comparison among 125I-chymotryptic digests of the 65 kDa AB8.28 and 55 kDa 3G8 molecules isolated from different tissues. The 65 kDa AB8.28 and the 55 3G8 molecules were isolated from the slab gel (Fig. 1) and digested with chymotrypsin. Chymotryptic digests were then analyzed on thin-layer cellulose plates by electrophoresis in the first dimension and chromatography in the second dimension. Panels A-D: peptide maps of the 65 kDa AB8.28 molecules precipitated from the LGL and WRMS-186 cell lysates and the 55 kDa 3G8 molecules precipitated from the LGL cell lysate.
Discussion

The receptor for IgG Fc region has been expressed on the surface of human leukocytes has been recently depicted as a family of molecules of previously unrecognized heterogeneity. By a general agreement based on multiple analytical criteria, these structures have been classified as FcRI, FcRII and FcR [31].

We recently described a molecule termed AB8.28 [15] exerting IgG Fc receptor activity and capable of transducing an epitope-mediated activation signal in LGL, followed by significant release of PAF. Initially this MoAb has been categorized as an anti-CD16: successively a detailed epitope and modulation analysis [17], along with phenotypic data of LGL leukemia cells [21], prompted us to evaluate the hypothesis that AB8.28 molecule may be different from CD16. For the purpose, we devised a comparative analysis between AB8.28 and 3G8 molecules, the latter recognized by an antibody used as reference for CD16.

The differences between the molecules above referred were confirmed by the results of SDS-PAGE of the 3G8 and AB8.28 immunoprecipitates, which showed distinct molecular masses and typical polydispersity for the 3G8 molecule which probably includes two poorly distinct bands. Another difference was found in the expression of these molecules on resting T cells, where (as expected) AB8.28 was not found at variance with 3G8 (its presence likely accounts for the expression on T-gamma subset), whereas MLR-activated cells expressed predominantly the AB8.28 structure. Moreover the same molecule was immunoprecipitated from T and B leukemias, as well as from the sarcoma, carcinoma and fetal fibroblast lines, where 3G8 was not detectable. The AB8.28 structure has not shown variation when immunoprecipitated from LGL, MLR cells, T and B leukemias or carcinoma lines. These findings were confirmed by the analysis of N-linked carbohydrates and by the results of IEF-SDS-PAGE, which gave identical pattern of the molecules purified from different sources. This may be confidently taken as firm indication that molecules bearing AB8.28 epitope are identical in different tissues.

The differences between AB8.28 and 3G8 molecules were further stressed by the result of the chymotryptic peptide mapping which gave patterns distinct both in peptide distribution and migration. This type of analysis using AB8.28 immunoprecipitate from different sources indicates without doubt that this receptor does not show structural and molecular variation in different lines.

However, with in mind that this analysis may amplify differences and underscore identities [32], we re-evaluate the same maps which consented us to identify a number of peptides shared by the AB8.28 and 3G8 molecules (arrowed in Fig. 3).

The conclusions drawn from this work could be summarized as follows:

1) the 65 kDa AB8.28 molecule, previously characterized as an FcR mainly expressed on LGL and granulocytes, actually displays a broader distribution, being detectable on alloactivated T cells, some T and B leukemia lines along with various non-lymphoid tumor cells;

2) SDS-PAGE, IEF-SDS-PAGE, endo-F chymotryptic peptide mapping of the AB8.28 FcR molecules expressed on normal and tumor cells suggested identity of these molecules, rather than expression of the same epitope on structurally different molecules;

3) LGL cells co-express at least two distinct FcR molecules: 65 kDa AB8.28 and a clearly distinct 55 kDa FcR. Similarity between these two molecules has been suggested by usual functional tests such as inhibition of antibody-coated erythrocyte rosette formation and binding of pre-formed immune complexes [9, 15];

4) At variance with 3G8, the AB8.28 molecule is present on a few cells and tissues which apparently seem to play definite roles in hemopoiesis or in non adaptive immunity.

A picture able to include in a unifying view all these findings should rely upon the postulate of the existence of a fourth FcR molecule, which is expressed on normal cells and tumor lines beside and, in some instances, contemporarily with the so far unknown receptors.

This molecule has an IgG FcR activity similar to that of FcR even if it is clearly distinct in term of molecular mass, biochemical characteristics and tissue distribution. The presence of this receptor in cells not directly involved in hemopoiesis or immunological functions (e.g. fibroblasts) appears to be intriguing; on the other side some evidence has been previously reported that molecules with FcR activity may be present on the surface of non lymphoid cells since various marine and human tumor cells were able to adsorb antibody-coated sheep erythrocytes [33]. Moreover, a molecule with the molecular mass similar to that of FcR and recognized by the MoAb VIF3 has been recently described on the surface of the human non-lymphoid tumor cell [24].

Functional importance of the AB8.28 FcR molecule expressed on such various tissues and tumor cells is unclear at the present time. In a simplifying model, the expression of this molecule appears to be linked to the proliferation and/or activation state of the cell independently of the linkage, and suggests a new function for the AB8.28 molecule as a broad activation marker. In case of LGL activation [19], the AB8.28 molecule might have a protagonist role, whereas in other cells the presence of this molecule on the surface is the result of ongoing activation processes. Alternatively, one can hypothesize that the AB8.28 molecule present on other than LGL cells acts as a receptor for unknown growth and differentiation factors, as suggested by the ability of this molecule to transduce degranulation signals in LGL or the release of substances enhancing macrophage/granulocytes colony formation. Further, one can hypothesize that AB8.28 precedes CD16 molecule in ontogenesis, as evidenced by its appearance in embryonic thymic and fetal cells and devise a model attributing to AB8.28 molecule a role as Fe proteorceptor from which CD16 structure may have diverged. As a memory of the
(putative) common origin, the latter receptor conserved its partial sharing constitutional peptides, IgG binding function and tissue distribution. A comparative analysis of the ability to trigger LGL cells activation and PAF release has been hampered by the unavailability of the 3G8 MoAb in insoluble form. Further functional and biochemical studies on the AB8.28 molecule expressed on nonlymphoid tissues will be helpful to elucidate this issue.

At this point, we can confidently conclude that the family of IgG FcR's may include a fourth member, arbitrarily designated as FeR IV following the model suggested by Anderson [31], whose summary has been modified as illustrated in Table 2.

Applications

It is known that cell surface bears not only antigen-specific receptors and so far a score of structures (some of them with receptorial activity) has been identified which may find a role as up- or down-regulators of the immune function in humans, since in appropriate instance it is possible to modulate either the receptor or the agent mimicking the natural ligand. As a consequence of this observation, we have tried to use the AB8.28 FeR to focus the activity of NK towards selected or pre-defined targets. The basic idea has been inferred from data obtained studying ultrastructural variations observed during activation of human natural killer cells [34]. In fact, it resulted that AB8.28-Sepharose is able to activate NK cells and that the cytolytic ability of both resting and lymphokine activated NK cells is virtually abrogated after the exposure to this insolubilized reagent. It has been shown that the observed effect is not secondary to the lack of conjugate formation between NK cells and target tumors, but to complete discharge of granules from AB8.28-treated LGL. The immediate consequence of this finding is that the LGL treated as above deliver around them the content of their granules. To focus this activity could prove of extreme interest, for one interested in building up a biological pharmaceutical based on this antibody. The strategy we are pursuing at the moment, is addressed to the construction of bifunctional monoclonal antibodies [35, 36] where the product of a hybrid hybridoma has one arm recognizing the FeR on the NK cells and the other one is specific for a molecule expressed on a selected target. For the purpose we are building up such reagents using retroviral vectors able to infect the parental hybridoma cells and carrying a dominant selection marker, which confers resistance to an inhibitor of the cell growth. The vectors used include pMV7neo [37], pN2neo, pSPDhfr [38], pMSVgpt [39], which permit the cells to survive in G418 genentic, mycophenolic acid and methotrexate respectively [35]. The reagents obtained are devised to

Table 2. - Human IgG Fc receptors

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>FeR I</th>
<th>FeR II</th>
<th>FeR III</th>
<th>FeR IV</th>
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<tr>
<td>Molecule</td>
<td>72 kDa</td>
<td>40 kDa</td>
<td>50-70 kDa</td>
<td>65 kDa</td>
</tr>
<tr>
<td>Distribution</td>
<td>monocytes, (HL-60, U937)</td>
<td>monocytes, neutrophils eosinophils, platelets B cells (U937, HL-60, K562, Daudi, Raji)</td>
<td>neutrophils, eosinophils macrophages, LGL, Tgamma</td>
<td>neutrophils, LGL Tgamma (lymphoid and carcinoma lines)</td>
</tr>
<tr>
<td>Affinity for monomeric IgG</td>
<td>$K_d = 10^8 - 10^9$ M$^{-1}$</td>
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<td>undetectable</td>
<td>not done</td>
</tr>
<tr>
<td>Sites/cells</td>
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<td>not done</td>
<td>not done</td>
</tr>
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<td>Anti-T3 proliferation</td>
<td>mlgG$\alpha$ (OKT3)</td>
<td>mlgG1 (Leu-4)</td>
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<td>Monoclonal antibodies</td>
<td>32</td>
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<td>3G8, 4F7, Leu-11a Leu-11b, VEP13, B73.1</td>
<td>AB8.28</td>
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<td>Ca$^{2+}$-dependence in transmembrane signaling</td>
<td>not done</td>
<td>not done</td>
<td>yes</td>
<td>no</td>
</tr>
</tbody>
</table>
constitute a trigger of NK cytolytic activity and simultaneously to focus it onto the target. These reagents may be built up on request, since the second arm may vary according to the individual need. At the moment, these reagents in construction are made between anti-FcR and CB30 (an anti-CEA MoAb) [40], which constitute an easy in vitro model, but other reagents are in preparation which include relevant targets as CALLA and other tumor markers, which may found eventually use in clinical experiments.

REFERENCES


