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Yeast cell surface display system for determination of humoral response to active immunization with a monoclonal antibody against EpCAM

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Abstract

Even though an immunogenic formulation of the murine monoclonal anti-EpCAM (epithelian cell adhesion molecule) antibody Mab 17-1A, has been shown to evoke a strong humoral immune response in both, monkey studies and early clinical trials, conventional anti-EpCAM ELISA could not identify anti-EpCAM immune response in relation to treatment with Mab17-1A. In contrast, usage of cellulose membranes prepared by SPOT technology presenting overlapping EpCAM peptides allowed the unequivocal determination of EpCAM related antibodies present in monkeys sera after immunization with IGN101. Based on such contradictory results, it was of high interest to compare obtained data to a different method for better assessment of their possible interpretation. Therefore, in the present studies, some EpCAM peptides, determined as reactive by binding of IgG isolated from sera of treated monkeys on membranes prepared by SPOT technology, were represented on yeast surface using the pYD1 yeast display vector system. Binding of biotinylated IgG from sera was detected with streptavidin–FITC and quantity of binding was determined by FACS measurement. Though using this completely different method, experiments with pre-immune and immune sera of four monkeys exemplarily are comparable to the results obtained by analysis with synthetic peptide arrays.

Keywords: EpCAM; ELISA; SPOT synthesis; Yeast surface display; FACS

1. Introduction

Since the epithelial cell adhesion molecule (EpCAM/GA733/KS1/4) is over-expressed in ~70% of epithelial cancers and their metastatic lesions it is an attractive target for monoclonal antibody immunotherapy. Recently IGN101, an active formulation of the murine Mab17-1A, originally designed to induce anti-EpCAM/anti-idiotypic antibody [anti-Id antibody] responses, has been shown to generate a strong anti-mouse immune response [1–6]. In contrast to Frodin et al. [7], induction of anti-EpCAM antibodies was not found using conventional ELISA. Therefore, a new methodology had been established to investigate the possible existence of anti-EpCAM antibodies and to study the relation of these antibodies with the vaccination [8]. This alternative method used overlapping synthetic peptides of high density covering the whole sequence of EpCAM prepared by SPOT synthesis on cellular supports to identify possible influence of vaccination via induction of EpCAM specific antibodies. Sera of healthy monkeys and cancer patients immunized with IGN101 versus sera of normal human donors were tested. For each individual IgG isolated from serum prior vaccination (pre-immune sera) and serum post-vaccination with IGN101 were analyzed for specific reactivity against these EpCAM peptides. Isolated IgG from sera

Abbreviations: EpCAM, epithelial cell adhesion molecule; Mab17-1A, murine monoclonal anti-EpCAM antibody; TAA, tumor-associated antigen; IgG, immunoglobulin G; HLA, human leukocyte antigen; Mab, monoclonal antibody; CTL, cytotoxic T lymphocytes; ELISA, enzyme-linked immunosorbent assay; Fmoc, 9-fluorenylmethoxycarbonyl; BSA, bovine serum albumin; PBS-T, phosphate-buffered saline containing 0.1%; Tween 20; HRP, horseradish peroxidase; Id, idiotype; FACS, fluorescence associated cell sorter.

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was directly labeled with biotin and binding to peptides was detected by incubation with streptavidin–horseradish peroxidase via chemiluminescence. This method was used to compare IgG from monkey sera and human sera pre-and post-immunization with IGN101, as well as IgG from healthy human donors by its binding to EpCAM peptides. It could be shown that sera from normal donors reacted with different peptides than sera from healthy monkeys, which indicates that some peptides may be recognized in a species specific manner. Cancer patients generally had a lower reactivity to EpCAM. Nevertheless, certain EpCAM peptides were determined to which binding of isolated IgG was shown to be specific and related to immunization with IGN101. These peptides again differed between human sera and monkey sera, but were still clustered in the same region of the protein.

Since these results were contradictory to that obtained by conventional ELISA, it was of high interest to compare them using an additional method. Representing a totally different methodology, the yeast surface display system was chosen as appropriate method. Due to lack of material it was decided to perform the cross-check of methods with monkey sera only. Therefore seven peptides, to which binding of isolated IgG from monkey sera was proved to be specific and related to immunization with IGN101, were expressed on *Saccharomyces cerevisiae* EBY100 using the pYD1 vector system. To ensure direct comparability with results obtained before, yeast cells were incubated with IgG isolated from monkey sera directly labeled with biotin. After incubation with streptavidin–FITC, bound antibodies were detected by FACS measurements. Differences of determined binding properties between pre-immune and immune sera again indicate the influence of immunization with IGN101.

2. Materials and methods

2.1. Sera

For the present study animal sera were obtained from 4 Rhesus monkeys (*Macacca mulatta*; animals 79 and 129 were females with body weights of 7.4 and 6.8 kg respectively; animals 264 and 331 were males with body weights of 5.2 and 4.3 kg respectively) prior immunization (pre-immune sera) and after (immune sera) immunization with IGN101 [$Al(OH)_3$ formulated Mab 17-1A], a murine IgG2a antibody specific for EpCAM.

2.2. Isolation and labeling of serum IgG

IgG was isolated from monkey or human sera via Protein G Sepharose™ 4 Fast Flow (GE Healthcare, Uppsala, Sweden). Bound IgG was eluted with 0.25 M acetic acid, pH 2.6, containing 20% (v/v) 1,2-ethanediol for stabilization of isolated IgG. Eluted IgG was directly collected into vials containing 1 M disodium carbonate aliquots to immediately raise the pH to 8.6. Afterwards, isolated IgG was labeled with biotinamidohexanoic acid *N*-hydroxysuccinimide ester (NHS-LC biotin, Sigma-Aldrich, St. Louis, MO, USA), dialyzed into phosphate-buffered saline (PBS), and stored at 4 °C after adding 0.2% sodium azide.

2.3. SPOT synthesis, membrane probing and evaluation of membranes

Cellulose-bound peptide libraries were semi-automatically prepared according to the method first published by Frank et al. [9] and slightly modified by Pflegerl et al. [10]. Each membrane (henceforth referred to as "EpCAM membrane") consists of 77 overlapping deca-peptides (with six-amino-acid *N*- and C-terminal overlaps in each peptide) covering the entire amino acid sequence of EpCAM (Swiss Prot accession number *P16422*). Membrane probing and regeneration were performed as described previously by Dürauer et al. [8]. For signal evaluation and interpretation the gray-scaled spots were converted into values using LumiImagerTM Software (Boehringer, Mannheim, Germany). Spots were localized by overlay of membranes with grid according to adjustment of SPOT synthesis. Calculated values were reduced by membrane background values. Spots #40 and #44 which had been identified as blank spots caused by binding of the detection agents, where taken as reference spots for intensity of signal. Ratio of signal intensity of these two spots on membrane used for determination of immune serum binding against their signal intensity on membrane used for determination of pre-immune serum binding was calculated for each individual. Determined values for binding to peptides were corrected with the calculated factor. Finally, ratio of corrected value for signal intensity on EpCAM membranes incubated with IgG isolated from immune serum against corrected value for signal intensity on EpCAM membranes incubated with IgG isolated from pre-immune serum was calculated and listed in Table 2. A ratio of 2 was accepted as significant different.

2.4. Strain and media

The yeast surface display strain EBY100 expressing the *AGA1* gene under control of the *GAL1* promotor was used for cell surface expression of selected EpCAM peptides. It was created by integrating the vector pIU21 into the *AGA1* locus of the *S. cerevisiae* strain BJ5465 (MATa *ura* 3-52 *trp* 1 *leu*2Δ1 *his*3Δ200 *pep*4:*HIS*3 *prp*1Δ1.6R *can*1 *GAL*) according to Boder and Wittrup [11]. The *Escherichia coli* strain genehogs (Invitrogen) was used for pYD1 gene cloning. Luria Bertani (LB) medium (10.0 g/L tryptone, 5.0 g/L yeast extract, 10.0 g/L NaCl, pH 7.5, supplemented with 50 μg/mL ampicillin) was used for bacteria growth and plasmid amplification. Untransformed EBY100 as well as transformants was grown on YNB-CAA medium containing 2% glucose (20.0 g/L glucose, 6.7 g/L yeast nitrogen base, 5.0 g/L casamino acids) and on YNB-CAA medium containing 2% galactose (with 20.0 g/L galactose replacing glucose) for induction.

2.5. Vector and cloning

pYD1 is a 5.0 kb expression vector designed for expression, secretion, and display of proteins on the extra-cellular surface of *S. cerevisiae*. Sense and anti-sense oligonucleotides were annealed and cloned into pYD1 using BamHI and NotI restriction