Frequent Antibody Production Against RARα

In Both APL Mice And Patients

Running title: anti-RARα antibodies in APL

Scientific heading: Neoplasia

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Abstract

In an acute promyelocytic leukemia (APL) transplantable mouse model, we previously reported the presence of antibodies recognizing PML-RARα and RARα in the sera of ATRA-treated mice. To evaluate this immune response, we determined the prevalence of anti-RARα antibodies in a cohort of 48 APL mice, treated by ATRA (n=24) or by placebo pellets (n=24), and in a preliminary subset of 9 APL patients using a specific Enzyme-Linked-Immunosorbent-Assay (ELISA). In APL mice, significantly higher antibody levels were observed at the latest time-points (days 48-58 levels superior to days 15-18 or days 28-38 levels). Antibody levels were higher in ATRA-treated mice than in placebo-treated mice and were also predictive of better survival. In the APL patients, anti-RARα antibodies were detected at diagnosis and after maintenance therapy, reminiscent of the ATRA-treated APL mice. Antinuclear or anti-neutrophil cytoplasmic auto-antibodies were also detected. These data reveal for the first time that in APL patients an immune response may be detected at diagnosis and enhanced after maintenance therapy.
Introduction

Acute promyelocytic leukemia (APL) is characterized by a differentiation block at the promyelocytic stage and by a reciprocal chromosomal translocation, t(15;17)(q24;q21) fusing the promyelocytic leukemia gene (PML) with the retinoic acid receptor alpha (RAR\(\alpha\)) gene\(^1\). Treatment with all-trans retinoic acid (ATRA) and chemotherapy induces complete remissions in 90% of PML-RAR\(\alpha\) positive APL patients through ATRA induced differentiation of the leukemic cells and their subsequent elimination by apoptosis\(^2\). Apart from their known effects on cell differentiation, retinoids also play an important role in infection and immune functions\(^3\). In hypovitaminosis A animal models and in vitamin A deficient children, alterations in antiviral, anti-bacterial or anti-parasite responses have been shown\(^3-5\).

In an APL transplantable mouse model which shares the promyelocytic features and response to ATRA of human APL\(^6\), we have previously reported the presence of antibodies in ATRA-treated-mice sera\(^7\). By western-blot analysis, we showed that these antibodies recognized mouse and human RAR\(\alpha\) from spleen and HL-60 cells respectively, as well as PML-RAR\(\alpha\) from mice spleen and NB4 leukemic cells. We hypothesized that such adjuvant effects of ATRA might also participate in the efficacy of ATRA in patients with APL especially during maintenance therapy when the tumor burden is low and immunocompetency restored\(^2\).

The aim of our study was therefore to further characterize anti-RAR\(\alpha\) antibody production in APL mice as well as in patients with APL. In APL patients, antibodies to
RARα and auto-antibodies like ANA (antinuclear auto-antibodies) or ANCA (anti-neutrophil cytoplasmic auto-antibodies) were analyzed.

Methods

The animal model of APL transplanted mice and ATRA therapy was set up as already described\(^6,7\). Data from three protocols which did not differ in their response to ATRA were pooled. Placebo (n=24) and ATRA-treated APL (n=24) mice died from day 18 to 29 (median 26 days), and from day 28 to 95 (median 55 days) respectively, as already reported\(^6,7\).

Antibodies against RARα were detected by an ELISA test at different time-intervals as previously described\(^7\). For each mouse serum, specific absorbance (SA) was calculated as the difference between duplicates of mean absorbance with and without GST-RARα. In order to normalize results between experiments, arbitrary units (AU) were obtained by dividing the SA obtained in serum dilution to the SA obtained with 1/200,000 dilution of positive control monoclonal anti-RARα antibody (9αF).

An ELISA test was performed in sera from 9 patients with APL to detect the presence of anti-RARα antibodies. Anti-nuclear (ANA) and anti-neutrophil cytoplasmic (ANCA) auto-antibodies were measured by indirect immuno-fluorescence. The 9 patients studied related to the availability of stored sera at diagnosis and after 2-year maintenance therapy\(^2\). Patients were enrolled in APL trials: patients 3,5,8 from APL93 and patients 1,2,4,6,7,9 from APL2000 trial. In these trials informed consent was obtained to use stored samples. For all patients sera were stored in similar conditions at -30°C and thawed once. 18 healthy subjects served as controls.
Results and discussion

Description of the anti-RARα antibody production in APL mice

As we have previously reported, anti-RARα antibodies were detected in ATRA-treated APL mice. Specificity of the ELISA signal defined previously on immunoblotting experiments with recombinant GST-RARα, was further assessed in this study by ELISA competition experiments. Pre-incubation of an anti-RARα positive serum overnight at 4°C with GST-RARα in a 20-fold excess, resulted in an 87% binding inhibition (specific absorbance of 0.158 compared to 1.23 with pre-incubation with buffer alone) (data not shown). The dilution of a positive anti-RARα mouse serum shows a dose-dependent specific absorbance similar to that obtained with a known anti-RARα monoclonal antibody 9αF8 (Figure 1a). In this large cohort of 48 APL mice, it is clear that anti-RARα antibody levels progressively increase with time, from a median AU level of 0.26 on days 15-18 (D"18"), to median AU levels of 0.88 on days 28-38 (D"38"), and 6.33 on days 48-58 (D"58") (t test, p=0.0008 between D"18" and D"58"). On D"38", among 6 surviving placebo-treated mice, only one mouse had antibody levels reaching a threshold of 4 fold that of the D"18" median, whereas in ATRA-treated mice 9/24 mice reached this level on D"38" and 12/13 on D"58", when all placebo-treated mice were dead (Figure 1b). AU reached a maximal value of 16.8 on D"58" in ATRA-treated mice. This antibody appearance followed the classical scheme of antibody production, with a time-decreased production of IgM and a time-increased production of IgG from day 18 to day 58 (Figure 1c). In order to investigate the prognostic value of these antibodies independently of time, we asked whether antibody levels obtained at D"18", when all mice were alive, were predictive of survival. At this time point, a higher level of antibody was associated with increased survival: median survival = 28 days in mice
with a level lower than the median D”18” AU (0.26), versus 49 days for mice with a level higher than the median D”18” AU (log-rang test, p=0.0319).

*Description of the anti-RARα antibody production in APL patients*

To provide relevance to the pre-clinical data obtained in APL mice, anti-RARα antibodies were retrospectively analyzed by ELISA in 9 APL patients. All patients were in complete remission at the time of post-maintenance treatment study. For the first time, anti-RARα antibodies were detected both at diagnosis and after maintenance therapy in APL patients, and the antibody production followed a time-dependent pattern similar to the kinetics of anti-RARα antibodies observed in APL mice (*Figure 1d and Table 1*). In 5 out of 9 patients anti-RARα antibodies levels increased with time. Of note, among the 4 patients in whom no increase in anti-RARα antibody levels was seen, 3 had already high levels of antibodies at diagnosis. Interestingly, other autoimmunity manifestations were observed in these patients. At diagnosis, seven (78%) were either positive for antinuclear (ANA) or anti-neutrophil cytoplasmic (ANCA) auto-antibodies and all patients were either ANA or ANCA positive after maintenance therapy (*Table 1*). This proportion was higher than that expected in the general population (13.3% and 0-1.8% of healthy persons may present ANA or ANCA auto-antibodies respectively). Thus APL patients present frequent auto-antibodies especially after maintenance therapy.

Antibodies against multiple tumor-associated antigens like oncogene proteins (myc, ras), gene suppressor proteins (p53), tumor antigens (MUC1, erbB2, WT1), or normal proteins (SPAN-Xb) have been described in patients with solid tumors or hematological malignancies. More recently, auto-antibodies have been shown to be markers of better prognosis in melanoma patients treated by interferon-alpha.
Our results show that in patients with APL, antibodies to RARα and auto-antibodies like ANCA, and especially ANA, are detected. An increase in anti-RARα antibodies and ANA was seen after maintenance therapy which includes ATRA and chemotherapy. In mice, as in humans, immunotherapy approaches have induced specific antibody production and in an APL patient vaccinated with an HLA-A2 restricted peptide PR1 derived from proteinase 3, PR1-tetramer-sorted CTLs were obtained after vaccination and showed lysis of patient’s bone marrow cells suggesting that an antitumor response may be elicited in APL. The data of our study supports the notion that an immune response elicited by APL cells is enhanced by ATRA therapy and may well be implicated in its efficacy as immunocompromised APL mice treated by ATRA have reduced survival. The immunogenic role played by ATRA in APL is not known and may be related to at least two mechanisms. One relates to the now recognized role of Vitamin A and its derivatives in the immune response, well seen in Vitamin A deficiency as in studies on Th2 response and dendritic cell activation triggered by retinoids. The other may relate to the direct effect of ATRA on the leukemic cell itself, where apoptosis and degradation of the oncoprotein PML-RAR could participate in cross-presentation. Ongoing prospective immunomonitoring of APL patients at diagnosis and throughout treatment will allow to establish whether the achievement of an efficient immune response is of prognostic value in APL and which criteria may be determinant. If the latter study demonstrates that antibody response correlates with disease free survival, it will provide, in the setting of minimal residual disease, the basis for vaccine boosting strategies that enhance a humoral and/or a cellular immune response.
Acknowledgements

We thank Cécile Rochette-Egly, IGBMC, Strasbourg, France for providing monoclonal anti-RARα antibody, Dr Felix Agbalika and Dr Catherine Scieux, Laboratoire de Virologie, Hôpital Saint-Louis, Paris, for providing some of the stored sera of APL patients, Scott Kogan and J. Michael Bishop, University of California, San Francisco, for the APL mouse cells, and Bernard Boursin, Service d’Infographie, IUH, Hôpital Saint-Louis, Paris, for excellent graphic work.

Supported by: the French Fondation pour la Recherche Medicale, the Fondation de France, the French Association de Recherche contre le Cancer, the French Ligue Nationale contre le Cancer, the Fondation Saint-Louis, Eli Lilly, INSERM and the Fondation de France.
References


### Table 1. Anti-RARα, anti-nuclear (ANA) and anti-neutrophil cytoplasm (ANCA) auto-antibodies in patients with APL

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**Abbreviations:** Ab = antibodies, ANA = Anti-Nuclear Auto-antibodies, ANCA = Anti-Neutrophil Cytoplasm Auto-antibodies.

* Diagnosis: Day 0 of therapy for all patients. ** Post-treatment: after maintenance therapy (delay** in months is given for each patient)

**Results of antiRARα antibodies** are expressed as semi-quantitative values according to specific absorbance (SA) value: SA<0.2 (+); SA = 0.2-0.4 (++, SA = 0.4-0.6 (+++), SA>0.6 (++++).

**Results of ANA and ANCA** are expressed as negative (-) if no fluorescence was observed, and as weakly positive (+) or strongly positive (++) according to the intensity of the signal. Both ANA and ANCA tests were performed according to standard routine procedures of the Laboratoire d'Immunologie by two independent investigators. Briefly, antinuclear auto-antibodies (ANA) were detected in the same sera (diluted 1/80) by indirect immunofluorescence with commercial Hep2000 cells (Immuconcepts, BMD). Anti-neutrophil cytoplasmic auto-antibodies (ANCA) were tested in sera (diluted 1/20) by indirect immunofluorescence with commercial human neutrophils (Inova Diagnostic, San Diego, CA, USA).
Legend to Figure 1

a) Comparative anti-RARα ELISA dose-response curves with a positive mouse serum and an anti-RARα mAb control

Specific absorbance measured by ELISA of a serum from an ATRA-treated mouse serially diluted from 1/50 to 1/10 000 (symbols = open triangles) and from the anti-RARα monoclonal antibody 9αF diluted from 1/10 000 to 1/200 000 (reference curve, symbols = open squares).

b) Time-dependent presence of anti-RARα antibodies in mice with APL

Anti-RARα antibodies were measured by ELISA in 48 mice with APL. On days 15-18, all mice were alive, sera were obtained from only 46 mice, including 23 mice treated by ATRA and 23 treated by placebo. On days 28-38: 30 mice were alive, including 24 mice treated by ATRA and 6 treated by placebo. On days 48-58, only 13 mice, treated by ATRA, were alive. The box plot in combination with dot plot displays a statistical summary of the data (quartiles and median). Data are expressed as arbitrary units (AU, see text).

c) Time course of the IgG and IgM specific absorbance in the anti-RARα ELISA of one ATRA-treated APL mouse

For IgG and IgM detection, secondary antibodies specific for mouse IgG and IgM were used in ELISA test. In one ATRA-treated mouse the anti-RARα ELISA was performed in serum collected on days 18, 28 and 38, using specific secondary antibodies for mouse IgG (symbols = filled circles) and mouse IgM (symbols = filled squares). Results are expressed as specific absorbance (SA).
d) Time-dependent presence of anti-RAR\textsubscript{\(\alpha\)} antibodies in APL patient sera

Anti-RAR\textsubscript{\(\alpha\)} antibodies (expressed as Specific Absorbance) were measured by ELISA in serum of 9 APL patients at diagnosis and after maintenance therapy. ELISA test was performed in 96 well-plates (Nunc, Merck Eurolab) coated with either GST or GST-RAR\textsubscript{\(\alpha\)} (obtained by fusion of a GST tag to the full length RAR\textsubscript{\(\alpha\)}). After overnight saturation with PBS1X-BSA 5%, sera (diluted 1/200 in PBS1X-BSA0.5 %) were incubated 1 hour at room temperature. Peroxidase-conjugated goat anti-human-IgG antibody (Sigma-Aldrich, Lyon, France) was added and incubated 1 hour at room temperature, followed by TMB substrate revelation (BD Pharmingen, San Diego, CA). Absorbance was measured at 450 nm (reference filter at 630 nm) using an optical densitometer (Dynatech MR5000, Labsystems, 95615 Cergy, France). For each serum, specific absorbance (SA) was calculated as the difference between duplicates of mean absorbance between GST-RAR\textsubscript{\(\alpha\)} and GST. Anti-RAR\textsubscript{\(\alpha\)} antibodies were also measured in sera from 18 healthy persons (controls). The box plot in combination with dot plot displays a statistical summary of the data (quartiles and median).
Frequent antibody production against RARα in both APL mice and patients