ANTINUCLEAR HUMAN AUTOANTIBODIES AS MARKERS IN *NICOTIANA TABACUM* POLLEN TUBES

POGGIALINI C.[1], MOROZZI G.[2], MARCOLONGO R.[2], VIGNANI R.[1], CRESTI M.[1], TIEZZI A.[3]

[1]Dipartimento di Biologia Ambientale,
Università di Siena, Via P. A. Mattioli 4, 53100 Siena, Italia

[2]Istituto di Reumatologia,
Università di Siena, Policlinico Le Scotte, Siena, Italia

[3]Dipartimento Scienze Ambientali,
Università della Tuscia, Via S. Camillo De Lellis,Viterbo, Italia

ABSTRACT

In the present paper we report on the use of antinuclear human autoantibodies as specific markers in *Nicotiana tabacum* pollen tubes. The antibodies have been tested by fluorescence techniques using a confocal laser scanning microscope. All the antibodies showed specifc labelling pattern and the results, although preliminary in nature, could open new perspectives of research.

KEY WORDS: Human autoantibodies, immunofluorescence, pollen tube, confocal microscopy.

INTRODUCTION

For research applications, antibodies are mainly obtained by biochemical purification of specific antigens and their subsequent injection in animal hosts in order to induce an immunological response. Depending on running improvement of technology, antibodies have become important and precise probes to identify and determine the location of molecules and molecular structures in both animal and plant cells.

It has been well established that in humans some pathologies move also to a production of specific antibodies and that these antibodies are markers of autoimmune diseases (Tan 1982, Tan et al. 1988). Systemic lupus erythematosus (SLE), mixed connective tissue diseases (MCTD), Sjogren's syndrome or scleroderma or systemic schlerosis, are presently diagnosted also by specific tests on these antibodies. The immune response of these patients includes for instance ANA antibodies (antinuclear antibodies) against their own antigens located in the nucleus and the nucleolus. Such specificity was tested by indirect immunofluorescence techniques.

Such antibodies opened new perspectives of research, allowing, in particular, investigations on polypeptides which are specifically located within the nucleus and playing there a crucial role. As an application, human antibodies from a patient with CREST syndrome, variant of scleroderma, have been shown to selectively stain kinetochores on mammalian chromosomes (Valdivia and Brinkley, 1985). Subsequently such antibodies were used in *Haemanthus katherinae* endosperm cells to detect the kinetochore organization during cell cicle (Mole-Bajer et al. 1990) and were also employed in *Tradescantia virginiana* to study kinetochores during generative cell division (Palevitz, 1990). The results revealed by human autoantibodies, confirmed their use as an useful tool of investigation.

Here we introduce the utilization of human autoantibodies in cytochemical immunolabeling in *Nicotiana tabacum* pollen tubes. These antibodies provides specific pattern of staining and might open interesting perspectives, contributing to the study of pollen biology and the applications for specific diagnostic purposes.

MATERIALS AND METHODS

Pollen culture

Pollen of *Nicotiana tabacum* L. was collected from plants grown in the Botanical Garden of the University of Siena. Pollen grains were dehydrated for 12 hours in a desiccater and then stored a -20°C. Before germination pollen grains were carefully thawed by an incubation for 15 minutes in ice and 15 minutes at room temperature. Pollen grains were rehydrated in a humid chamber for 1 hour and then cultured at 23-24°C in BK mediurn (Brewbaker and Kwack, 1963) containing 15% sucrose.

Fixation and enzyme treatment of pollen tubes

Samples were collected after about 3 h of germination and fixed in 3% paraformaldehyde in PEM buffer (100 mM PIPES pH 6.8, 1 mM $MgCl_2$ and 2 mM EGTA) containing 15% sucrose for 30 minutes. Samples were washed three times in PEM buffer containing 15% sucrose for 5 minutes. Pollen and pollen tubes were incubated with 2% cellulysin in PEM buffer

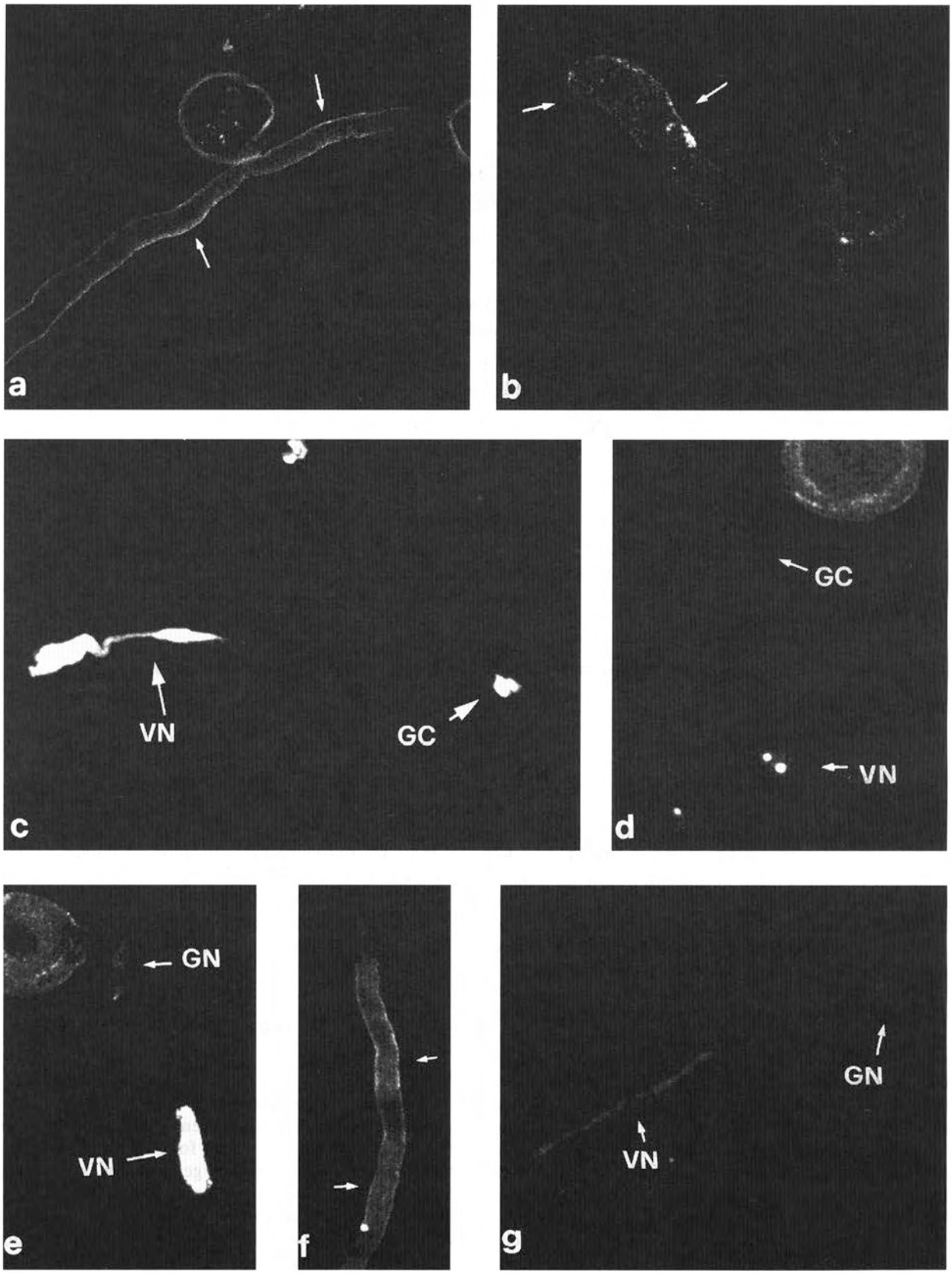

Fig. 1. Confocal laser microscopy of *Nicotiana tabucum* pollen tubes stained with different human autoantibodies (x 300).
a – anti SSB antibody. The antibody stains the wall (arrows) all along the lenght of the pollen tube.
b – anti SSA antibody. The antibody stains the pollen tube wall (arrows). A punctate staining is also present in the cytoplasm.
c – anti homogeneous nucleus antibody. The antibody selectively stains the vegetative nucleus (VN) and the generative cell (GC) nucleus.
d – anti nucleolar antibody. Nucleoli of the vegetative nucleus (VN) are stained and the vegetative nucleus shows a weak diffuse staining. Generative cell (GC) presents only a diffuse weack staining.
e – anti RNP antibody. The antibody stains both the vegetative nucleus (VN) and generative cell (GC) with different intensity.
f – anti RNP antibody. Staining of pollen tube wall (arrows).
g – anti Smith antibody. The antibody selectively stains both the vegetative nucleus (VN) and the generative cell nucleus (GN).

TABLE 1. Specificities and pathologies of the human autoantibodies (Tan et al., 1988).

Antibodies	Abbreviation	Specificities	Pathology
Anti-SSB	**SSB**	Phosphoprotein of48Kda associated with polymerase III transcripts	Sjogren's syndrome Systemic lupus erythematosus (SLE)
Anti-SSA	**SSA**	Small nuclear or cytoplasmic ribonucleoproteins	Sjogren's syndrome Systemic lupus erythematosus (SLE)
Anti-homogeneous nucleus		Nuclear proteins	Systemic lupus erythematosus (SLE)
Anti-nucleolar		RNA polymerase I	Scleroderma
Anti-RNP	**RNP**	Proteins of 33 and 22 Kda associated with U1 RNA	Mixed connective tissue diseases (MCTD)
Anti-Smith	**Sm**	Proteins associated with U1, U2, U4, U5, U6 RNA	Systemic lupus erythematosus (SLE)

for 3 minutes at room temperature in the dark and then rinsed three times in PEM buffer for 5 minutes. Pollen and pollen tubes were treated with methanol at -20°C for 3 minutes and then rinsed three times in PEM buffer for 3 times per 5 minutes and processed for immunofluorescence microscopy.

Immunofluorescence microscopy

Pollen tubes were fixed and processed for immunolabeling as previously reported (Del Casino et al., 1993). Samples of pollen and pollen tubes were incubated (at 4°C overnight) with the following primary antibodies, respectively: commercially available control, antihomogeneus nucleus, anti nucleolar, anti RNP, anti Sm, anti SSA, anti SSB (Immunoconcepts Sacramento, CA, USA. For further information on the antibodies see Table 1). All the antibodies were used without any dilution.

After several washings in PBS buffer (Phosphate Buffered Saline pH 7.4), the samples were incubated with a FITC-conjugated human IgG secondary antibody (Sigma), diluted 1:60 in PBS for 2 hours at room temperature. After some washings in PBS, the samples were mounted on slides and then observed with a Confocal Laser Scanning Microscopy (model MRC 500, Bio-Rad Instruments, Hemel Hempstead, UK), equipped with an argon ion laser and mounted on a Nikon Optiphot microscope. A 60 X objective and the BHS filterset were used for imaging. All images were obtained by using a stepper motor to make Z-series. The option XZ command was used for the reconstruction of optical-sections of the pollen tubes. Photographs were taken from the monitor on Kodak Tmax films (100 ASA).

RESULTS

All the tested antibodies reveal a selective staining *in Nicotiana tabacum* pollen tubes and the results are reported in Fig. 1. The controls gave negative results (data not shown).

Fig. 1a shows the pattern of staining of the antibody SSB. The antibody stains the wall all along the lenght of the pollen tube. The staining is selective as the cytoplasm and cytoplasmic organelles are not labelled. As the staining is uniform, the distribution of the epitope within the wall is homogeneous.

Fig. 1b shows the pattern of staining of the antibody SSA. The staining of this antibody is similar to the staining showed by the antiboby SSB. SSA antibody stains the wall all long the tube in a punctate way and a weak punctate staining is also visible within the cytoplasm.

In Fig. 1c the pattern of staining of the antibody anti homogeneous nucleus is reported. This antibody selectively stains both the nuclear materials of the vegetative nucleus (VN) and of the generative cell (GC). The staining is homogeneous and the antibody does not allow to discriminate between the two nucleus as the epitope seems to be equally expressed and distributed.

Fig. 1d shows the pattern of staining of the antibody anti nucleolus. The staining is very selective and clearly put in evidence two nucleoli within the vegetative nucleus (VN) in which a weak diffuse staining is visible. Nucleoli of the generative cell (GC) are not stained and the generative cell nucleus presents only a weack diffuse staining.

In Figs 1e and 1f the pattern of staining of the antibody anti RNP is reported. About 70% of investigated pollen and pollen tubes display the staining pattern showed in Fig. 1e. The antibody is specific for nuclear material and both the vegetative nucleus (VN) and the generative cell nucleus (GN) are stained. However as clearly showed by the intensity of staining, a different expression of the epitope within the two nuclei occurs. About 30% of the investigated pollen tubes present the staining pattern of Fig. 1f. This pattern of staining is completely different respect to that reported in Fig. 1e and resembles the pattern of staining of Fig. 1b. The staining of the pollen tube wall and a weak punctate staining of the cytoplasm are infact visible.

Fig. 1g shows the pattern of staining of the antibody anti Smith. The antibody is selective for nuclear materials. Infact both the vegetative nucleus (VN) and the generative cell nucleus (GN) are stained by this antibody. The pattern of staining resembles that of the anti homogeneous nucleus (Fig. 1c), although in this case within both nuclei the epitope is not uniformly distributed and zones showing different fluorescence intensity occur.

DISCUSSION

In the last years our knowledge on pollen biology greatly improved mainly depending by the use of specific chemical markers and antibodies. For instance the study of pollen and pollen tube cytoskeleton got consistent benefits and in recent time a set of ATPase proteins named "molecular motors" have

been identifted and characterized within the pollen tube by the use of specifc antibodies (Cai et al. 1993; Moscatelli et al., 1995; Tirlapur et al., 1995, Miller et al., 1995).

The use of human autoantibodies in plant research (Mole-Bajer at al., 1990) and in particular on pollen tube (Palevitz, 1990) already provided interesting results. The results showed here contribute in enlarging knowledge on pollen biology and confirm the use of such antibodies as useful tools of investigation. All the antibodies showed here present a marked specificity to nuclear antigens when tested in animal cells. In pollen tubes, such antibodies offer two distinct pattern of staining. Some antibodies (anti homogeneous nucleus, Fig. 1c; anti nucleolar Fig. 1d; anti Smith, Fig. 1g) show a selective specificity for nuclear antigens, other antibodies (anti SSB, Fig. 1a; anti SSA, Fig. 1b; anti RNP, Figs 1e, 1f) apparently changed their specificity offering a selective staining of pollen tube wall.

The antibodies showing specificity to nuclear antigens are selective tools of investigations. They stain nuclear materials and allow to put in clear evidence both the Vegetative Nucleus and the Generative Cell Nucleus. The antibody anti nucleolus is very specific for the nucleoli of the Vegetative Nucleus; in the Generative Cell Nucleus this antibody presents only a diffuse weak staining suggesting the absence of the antigen within the Generative Cell nucleoli. Such antibodies could find application in further detailed studies concerning the study of the nuclear material organization during generative cell division and sperm cells formation.

The antibodies showing a changed pattern of staining respect to that showed in animal cells need of additional investigation. Antibodies anti SSA and SSB clearly stain in a selective way the wall all along the lenght of the tube; anti RNP presents a double pattern of staining, showing in some cases a good specificity for nuclear materials and in other cases a staining pattern resembling those of anti SSA and SSB antibodies. It is difficult to explain why such antibodies changed their pattern of staining and why an antigen that in animal cells is located within the nucleus, in pollen tube can be present in the pollen tube wall. Probably the epitopes could be part of proteins or glycoproteins specifically located within the wall. Anti SSA and anti SSB could allow in future further studies concerning the biogenesis of the wall during pollen tube growth. Depending on double labelling pattern, anti RNP does not offer good perspective for further investigation.

The results showed here, although preliminary in nature, open also perspectives for the application of pollen tubes as unexpensive substrate for diagnostic purposes. Further studies are running in order to establish the pattern of staining of other human autoantibodies in *Nicotiana tabacum* pollen tube.

ACKNOWLEDGEMENTS

We wish to thank Prof Yi-Qin Li for useful comments during preparation of the manuscript and Mr. Fabrizio Ciampolini for technical help.

LITERATURE CITED

BREWBAKER J.L., KWACK B.H., 1963. The essential role of calcium ion in pollen germination and pollen tube growth. Am. J. Bot., 50, 859-865.

CAI G., BARTALESI A., DEL CASINO C., MOSCATELLI A., TIEZZI A. and CRESTI M., 1993. The kinesin-immunoreactive homologue from *Nicotiana tabacum* pollen tube: biochemical properties and subcellular localization. Planta, 191, 496-506.

DEL CASINO C., LI YI-QIN, MOSCATELLI A., SCALI M., TIEZZI A. and CRESTI M., 1993. Distribution of microtubules during the growth of tobacco pollen tubes: Biol. Cell., 79, 125-132.

MILLER D.D., SCORDILIS S.P., HEPLER P.K., 1995. Identification and localization of three classes of myosins in pollen tubes *of Lilium longiflorum* and *Nicotiana alata*. J. Cell Sci., 108, 2549-2553.

MOLE-BAJER J., BAJER A.S., ZINKOWSKI R.P., BALCZON R.D., and BRINKLEY B.R., 1990. Autoantibodies from a patient with scleroderma CREST recognized kinetochores of the higher plant *Haemanthus*. Proc. Natl. Acad. Sci. USA, 87, 3599-3603.

MOSCATELLI A., DEL CASINO C., LOZZI L., CAI G., SCALI M., TIEZZI A. and CRESTI M., 1995. High molecular weight polypeptides related to dynein heavy chains in *Nicotiana tabacum* pollen tubes. J. Cell. Sci., 108, 1117-1125.

PALEVITZ B.A.,1990. Kinetochore behavior during generative cell division in *Tradescantia virginiana*. Protoplasma, 157, 120-127.

TAN E.M., 1982. Autoantibodies to nuclear antigens, Their immunobiology and medicine. Adv. Immunol., 33, 167-240.

TAN E.M., CHAN E.K.L., SULLIVAN K.F, RUBIN R.L., 1988. Antinuclear antibodies (ANAs): diagnostically specific immune markers and clues toward the understanding of systemic autoimmunity. Clin. Imm. Immunopathol., 47, 121-141.

TIRLAPUR U.K., CAI G., FALERI C., MOSCATELLI A., SCALI M., DEL CASINO C., TIEZZI A. and CRESTI M., 1995. Confocal imaging and immunogold electron microscopy of changes in distribution of myosin during pollen hydration, germination and pollen tube growth in *Nicotiana tabacum* L., Eur. J. Cell Biol., 47, 209-217.

VALDIVIA M.V., BRINKLEY B.R., 1985. Fractionation and initial characterization of the kinetochore from mammalian metaphase chromosomes. J. Cell Biol., 101, 1124-1134.

UŻYCIE AUTOPRZECIWCIAŁ CZŁOWIEKA DO WYKRYWANIA ŁAGIEWEK PYŁKOWYCH *NICOTIANA TABACUM*

STRESZCZENIE

Przeiwjądrowe przeciwciała człowieka mogą być użyte jako specyficzne markery łagiewk pyłkowych *Nicotiana tabacum*. Badania przeprowadzono technikami fluorescencyjnymi z użyciem skaningowego mikroskopu konfokalnego. Przeciwciała wiązały się specyficznie, a wstępne wyniki wydają się otwierać nowe perspektywy badawcze.

SŁOWA KLUCZOWE: przeciwciała człowieka, immunofluoroscencja, łagiewka pyłkowa, mikroskopia konfokalna.