# Study of the Greening Organism (GO) With Monoclonal Antibodies: Serological Identification, Morphology, Serotypes and Purification of the GO

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ABSTRACT. A monoclonal antibody (MA) previously developed to a strain of the greening organism (GO) from Poona, India, was used to purify the homologous organism from infected periwinkles. Immunofluorescence and immunogold labeling were used to identify the GO before and after purification. Isolated cells of the GO could be observed for the first time in the electron microscope. Both filamentous forms and round forms were seen. The filamentous forms were 1 to 4 μm. The diameter of their cross section varied between 0.1 and 0.3 μm. The round forms had a diameter of 0.2 to 1 μm.

We have also prepared several MA against a strain of the GO from Fujian province, China. These MA were able to detect the homologous GO strain but not the Indian strain of the GO. The MA against the Indian strain detected the homologous Indian strain as well as strains from Assam, Delhi and Shillong (India), Bali and Java (Indonesia) and Lipa city (Phillipines); they did not detect strains from China, Thailand and Malaysia; several strains from India, Indonesia and the Phillipines. These results demonstrate for the first time that different stereotypes of the GO exist, even within the same geographical area. Preparation of polyclonal antibodies and DNA probes less specific than MA is in progress to detect all GO strains.

Greening disease of citrus is characterized by the presence of bacteria-like organisms (BLO) localized exclusively in the sieve tubes of infected plants (6). Morphologically speaking, the same BLO if found associated with greening disease from all geographical origins (Africa, Asia, Arabic peninsula). Even though Koch's postulates have not been fulfilled, these BLOs are considered to be the causal agent of the disease. In this paper we report the production of monoclonal antibodies (MA) against the GO from Fujian (China) and the use of such MAs as well as those prepared previously against the GO from Poona (India), for serological studies of the organism.

## MATERIALS AND METHODS

**Production of monolocal antibodies.** The procedures for the production of screening of GO-specific hybridomas has been described (4).

**Nature of the antigens recognized by monoclonal antibodies.** The nature of the GO antigens carrying the epitopes recognized by the MAs was determined by immunofluorescence (IF) on midrib sections of GO-infected periwinkles previously submitted to one of the following treatments: i) heating at 100C for 5 min; ii) digestion with proteinase K (0.5mg/ml) for 90 min at 37C; iii) treatment with 50mM periodate in PBS for 60 min at room temperature (RT) in the dark; iv) treatment with 1M NaCl for 30 min at RT. Each treatment was followed by extensive washing with phosphate buffer saline (PBS 0.1M pH 7.0). The sections were then incubated for 30 min at RT with the various hybridoma supernatants, rinsed with PBS-tween (PBS plus 0.05% tween 20) and incubated with fluorescein isothiocyanate (FITC)-labeled goat anti mouse immunoglobulins (Diagnosis Pasteur) for 30 min at RT. After washing with PBS-tween, the sections were mounted in 50% glycerol in PBS pH 7.4 and observed in a Zeiss III RS epifluorescent microscope (filter combination BP 455-490/ FT 510/ LP 420).

**Electron microscopy of the GO in periwinkle extracts and in purified preparations.** The collodion transfer technique (3) with or without immunogold-labeling was used for GO observation and characterization.

**Immunoaffinity purification of**

**Poona-GO**. Antibody 10A6 has been coupled to CNBr activated Sepharose 4B and used to purify Poona-Go by immunoaffinity. The organisms were eluted from the matrix by 0.1M glycin at pH 11.5. A detailed description of the procedure is presented elsewhere (7).

**Double sandwich ELISA (ds Elisa) for the detection of Poona-GO**. The two hybridomas 2D12 and 10A6 were labeled with alkaline phosphatase (AP) and then used to develop a DAS-ELISA as described by Clark and Adams (1).

## RESULTS

**Hybridomas against Poona (India) and Fujian (China) greening organisms.** The production of two hybridomas (2D12 and 10A6) against GO from Poona has already been described (4). Using the same procedure, we have been able to produce six hybridomas (10F4, 2H9, 5H10, 11H6, 12E12, 6G1) secreting immunoglobulins against the Fujian-GO. The hybridomas were selected using the two assays previously described: differential avidin-biotin ELISA and immunofluorescence (IF) on sections of healthy and GO-infected periwinkle midribs (4). The two Poona-GO specific hybridomas gave positive reactions with GO-infected material in both assays. Four (2H9, 6G1, 11H6, 12E12) of the Fujian-GO specific hybridomas gave a strong, positive IF reaction in the phloem of Fujian-GO infected periwinkle midribs but did not react with GO infected plant tissue in the ELISA assay. The various hybridomas (Poona-GO and Fujian-GO specific) were characterized for the nature of the immunoglobulins produced and for the nature of the GO-epitopes recognized (Table 1). Various immunoglobulin classes (IgG and IgM) and isotypes (G1, G2a, G2b, G3) have been obtained. As far as the epitopes are concerned, all hybridomas except 10F4 are specific for epitopes that are temperature sensitive. Some immunoglobulins (2D12, 10A6, 2H9, 12E12, 6G1) do not react after treatment of the sections with proteases while other does (10F4 and 5H10). Hybridoma 2H9 is the only one that does not react when the sections are treated with 1M NaCl. The epitopes recognized by hybridomas 2H9, 5H10, and 11H6 are sensitive to the action of periodate. The epitope recognized by 10F4-IgG3 was insensitive to all the treatments applied. Western blots (Fig. 1) indicate that hybridomas 10A6 and 2D12 both rec-

TABLE 1
CHARACTERIZATION OF POONA-GO AND FUJIAN-GO SPECIFIC MONOCLONAL ANTIBODIES

| Hybridoma | Origin of the GO Strain | Isotype | Immunofluorescence in the phloem of GO-Infected periwinkles after the following treatments: | | | | |
|---|---|---|---|---|---|---|---|
| | | | None | 100C 5 min | Protease | NaCl 1M | Periodate |
| | India | | | | | | |
| 2D12 | Poona | IgG2a | +[z] | – | – | ND | ND |
| 10A6 | Poona | IgG1 | + | – | – | ND | ND |
| | China | | | | | | |
| 2H9 | Fujian | IgG1 | + | – | – | – | – |
| 10F4 | Fujian | IgG3 | + | + | + | ND | + |
| 5H10 | Fujian | IgG2b | + | – | + | + | – |
| 11H6 | Fugian | IgG1 | + | – | + | + | – |
| 12E12 | Fujian | IgG1 | + | – | – | + | + |
| 6G1 | Fujian | IgM | + | – | – | + | + |

[z] + = positive immunofluorescence; – = no immunofluorescence; ND = not done.

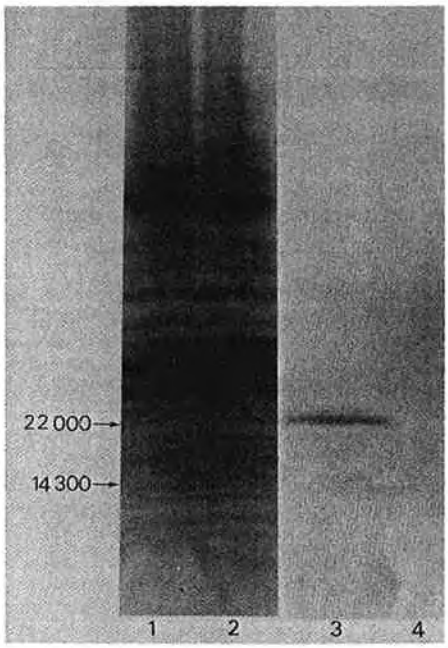


**Fig. 1. Polyacrylamide gel electrophoresis (1,2) and western blot (3,4) of proteins extracted from healthy (1,4) and Poona-GO-infected (2,3) periwinkle midribs. A mixture of 2D12 and 10A6 monoclonal antibodies was used to label the GO-specific band on the western blot.**

ognized a GO-protein of about 22,000d. IF and immunogold labeling (see below) of the GO indicate that all the epitopes involved were exposed on the surface of the organism.

**Developmental of double antibody sandwich ELISA (DAS-ELISA) for the detection of GO using various combinations of MAs.**

*Detection of Poona-GO.* PBS buffer and homogenates of healthy periwinkle midribs (HP) were used as negative controls. Table 2 shows that the highest optical densities (OD) were obtained with GO-infected extracts (IP) using MA 2D12 as the coating antibody and alkaline phosphatase-conjugated MA 10A6. In all assays, OD close to zero were obtained with PBS buffer and healthy extracts. We have subsequently used a DAS-ELISA for the detection of Poona-GO with coating antibody 2D12 at 10 μg/ml and the AP-conjugated antibody 10A6 at a 1/800 dilution. Using such an assay, series of ten healthy and infected periwinkle plants and citrus seedlings from the greenhouse were tested. All Indian-GO-infected periwinkles gave a strong positive reaction (OD>2) while in the case of citrus the OD varied between 0.2 and >2. The extract from the most heavily infected citrus (OD>2) gave a positive reaction even when diluted 256 fold. No positive reactions were observed with any of the healthy citrus or periwinkles tested.

*Detection of Fujian-GO.* DAS-ELISA was also developed for the detection of Fujian-GO with antibodies 10F4 and 2H9. When 10F4 MA was used both for coating and for the conjugate, the maximum OD of the reaction obtained with infected periwinkles was about 0.8 while it was >2 when 2H9-IgGs were used in the same way. When 2H9 antibody was used in DAS-ELISA, the periwinkle extract containing the GO antigen gave a positive reaction when diluted up to 512 fold. It has to be noticed that because the protein recognized by 2H9 was detached from the GO by 1M NaCl, the midrib homogenate was prepared in MOPS buffer (MOPS 50mM,pH 7.4, PVP 6%, EDTA 5 mM, Ascorbic acid 17.6 mg per 100 ml, PMSF 100 μM, pH 7.4) instead of PBS. DAS-ELISA performed with combinations of 10F4 and 2H9 antibodies gave poor results. A DAS-ELISA has also been developed with antibodies 11H6 and 5H10. The highest OD (>2) with GO-infected periwinkle was obtained when 11H6 was used for coating and 5H10 as the AP-conjugate. Other combinations for the detection of Fujian-GO will be tested.

**Detection of GO from various geographical areas with Poona-GO and Fujian-GO specific antibodies in periwinkles and citrus.**

Detection of GO from various geographical areas was studied by DAS-ELISA and IF with Poona-GO specific hybridomas and by IF for Fujian-GO specific hybridomas. Results

TABLE 2
DAS - ELISA FOR THE DETECTION OF POONA-GO

| Monoclonal antibodies used for | | | Alkaline phosphatase - conjugate dilutions | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1/500 | | | 1/800 | | | 1/1000 | | |
| | | | Antigens | | | | | | | | |
| Coating | Alkaline phosphataes conjugate | IgG Concentration for coating | a[z] | b | c | a | b | c | a | b | c |
| 10A6 | 10A6 | 5 | 0.008 | 0.046 | 0.334 | 0.005 | 0.020 | 0.271 | 0.008 | 0.025 | 0.225 |
| | | 10 | 0.002 | 0.046 | 0.563 | 0.005 | 0.020 | 0.502 | 0.005 | 0.014 | 0.480 |
| | | 15 | 0 | 0.031 | 0.657 | 0.005 | 0.020 | 0.650 | 0 | 0.008 | 0.605 |
| | | 20 | 0.009 | 0.031 | 0.900 | 0.010 | 0.040 | 0.840 | 0.003 | 0.050 | 0.740 |
| 2D12 | 2D12 | 5 | 0.026 | 0.026 | 0.445 | 0.015 | 0.030 | 0.441 | 0.015 | 0.020 | 0.385 |
| | | 10 | 0.015 | 0.020 | 0.650 | 0.015 | 0.020 | 0.574 | 0.010 | 0.020 | 0.460 |
| | | 15 | 0.014 | 0.025 | 0.738 | 0.010 | 0.009 | 0.570 | 0.015 | 0.025 | 0.465 |
| | | 20 | 0.019 | 0.030 | 0.850 | 0.006 | 0.008 | 0.683 | 0 | 0.023 | 0.473 |
| 10A6 | 2D12 | 5 | 0.010 | 0.025 | 0.255 | 0.020 | 0.008 | 0.214 | 0.009 | 0.026 | 0.193 |
| | | 10 | 0.010 | 0.020 | 0.549 | 0.013 | 0.006 | 0.606 | 0.011 | 0.015 | 0.483 |
| | | 15 | 0 | 0.015 | 0.700 | 0 | 0.010 | 0.694 | 0.017 | 0.008 | 0.657 |
| | | 20 | 0.030 | 0.025 | 0.850 | 0.008 | 0.035 | 0.800 | 0.026 | 0.007 | 0.750 |
| 2D12 | 10A6 | 5 | 0.007 | 0.022 | 1.242 | 0.010 | 0.023 | 1.056 | 0.008 | 0.020 | 0.850 |
| | | 10 | 0.015 | 0.024 | >2 | 0.003 | 0.017 | >2 | 0.020 | 0.012 | >2 |
| | | 15 | 0.015 | 0.013 | >2 | 0.014 | 0.018 | >2 | 0.010 | 0.008 | >2 |
| | | 20 | 0.025 | 0.034 | >2 | 0.042 | 0.027 | >2 | 0.015 | 0.026 | >2 |
| 10A6 2D12 | 10A6 2D12 | 5 | 0.050 | 0.040 | 0.838 | 0.040 | 0.040 | 0.640 | 0.030 | 0.030 | 0.580 |
| | | 10 | 0.030 | 0.025 | >2 | 0.008 | 0.025 | >2 | 0.007 | 0.013 | >2 |
| | | 15 | 0.030 | 0.040 | >2 | 0.014 | 0.035 | >2 | 0.015 | 0.030 | >2 |
| | | 20 | 0.030 | 0.070 | >2 | 0.020 | 0.049 | >2 | 0.015 | 0.045 | >2 |

[z] OD at 405 nm; a = PBS buffer control; b = healthy periwinkle midribs; c = Poona GO-infected periwinkle midribs.

in table 3 indicate that positive reactions were obtained in periwinkle plants and citrus seedlings infected with the homologous strain of the GO. However in most of the cases, no reactions were obtained with GO from other geographical areas. The two MAs specific for Poona-GO were able to detect GO from various areas in India (Poona, Assam, Delhi, Shillong) but GO from several other areas did not react. Philippines-GO was detected in a Lipa City isolate kept by repeated graft-inoculation in Bordeaux since the 1970's, but Philippines-GO tested in 1988 in Lipa City did not react with Poona-GO specific monoclonal antibodies. Antibody 10A6 but not 2D12 could detect GO from Indonesia. This shows that 10A6 and 2D12 antibodies recognize two different epitopes on the 22,000 dalton antigenic protein of Poona-GO.

Fujian-GO specific MAs have been tested only with greenhouse kept plants. They reacted with the homologous strain only, except for hybridoma 5H10 which gave a positive reaction with Taiwan-GO (likubin), an isolate obtained in 1969. No recent isolates have been tested.

**Use of MAs for morphological studies and purification of the GO.**

*Morphology of the GO in plant extracts.* As of today, GO has been observed only in phloem from infected periwinkles or citrus. Two forms of the organism have been described: elongated forms and round ones (2) but their size could not be precisely determined on thin sections. Using either IF or EM (2b), we were able to

TABLE 3
DETECTION OF GO OF VARIOUS GEOGRAPHICAL ORIGINS WITH POONA AND FUJIAN GO SPECIFIC MAs IN PERIWINKLE AND CITRUS PLANTS

| Antigens | Antibodies to Poons strains[z] 2D12 | 10A6 | Fujian strains[y] 2H9 | 10F4 | 5H10 | 11H6 | 12E12 | 6G1 |
|---|---|---|---|---|---|---|---|---|
| Periwinkles: | | | | | | | | |
| Healthy | –/– | –/– | – | – | – | – | – | – |
| Infected with GO from: | | | | | | | | |
| •India (Poona) | +/+ | +/+ | – | – | – | – | – | – |
| • China (Fujian) | –/– | –/– | + | + | + | + | + | + |
| • South Africa (Nelspruit) | –/– | –/– | – | – | – | – | – | – |
| • Philippines (Lipa City) | –/– | –/– | – | – | – | – | – | – |
| Citrus: | | | | | | | | |
| Healthy | –/– | –/– | – | – | – | – | – | – |
| Infected with GO from: | | | | | | | | |
| • India Poona | +/+ | +/+ | – | – | – | – | – | – |
| Assam | +/+ | +/+ | ND | ND | ND | ND | ND | ND |
| Shillong | +/+ | +/+ | ND | ND | ND | ND | ND | ND |
| Delhi | +/+ | +/+ | ND | ND | ND | ND | ND | ND |
| • Indonesia (Bali) | –/– | +/+ | – | – | – | – | – | – |
| • Thailand (Nakhom Pathom) | –/– | –/– | – | – | – | – | – | – |
| • China (Fujian) | –/– | –/– | + | + | + | + | + | + |
| • Taiwan (1969) | –/– | –/– | – | – | + | – | – | – |
| • Philippines (Lipa City) 1970 | +/+ | +/+ | – | – | – | – | – | – |
| • Philipines (Lipa City) 1988 | –/ND | –/– | ND | ND | ND | ND | ND | ND |

[z]ELISA reaction/IF reaction
[y]IF reaction only; ND = not done.

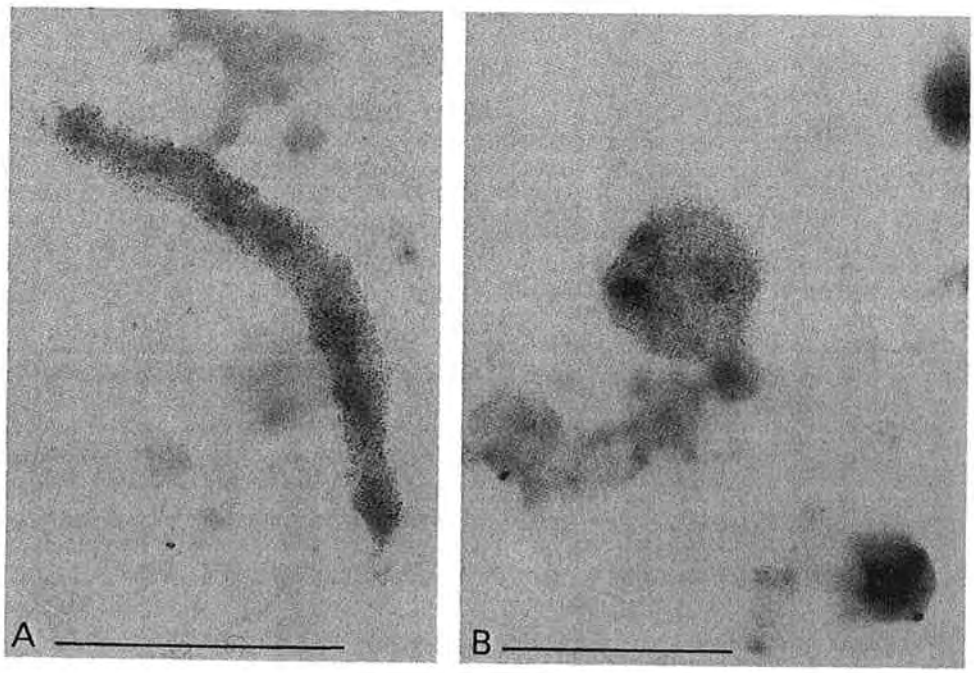


**Fig. 2. Immunogold labeling of filamentous (A) and round (B) forms of Poona-GO in periwinkle extracts with 10A6 monoclonal antibody. Bars = 1μm.**

observe Poona and Fujian GOs in infected periwinkle extracts. The two forms can be observed with both techniques, however for EM it was necessary to apply immunogold labeling to distinguish the round forms of the GO from plant debris of similar morphology. Gold-labeled elongated and round forms of Poona-GO are shown on fig. 2. Length of the elongated forms varied from 1 μm to more than 4 μm with an average around 2 μm (Fig. 3). The diameter of the round forms was between 0.3 μm and 1 μm. The morphology and size of Poona and Fujian GOs were similar.

*Immunoaffinity purification of Poona-GO.* Fig. 4 shows immunopurified GOs with or without gold-labeling with 10A6 MA. The morphological and serological properties of the GO after purification were comparable to those of GO in plant tissues and plant extracts as illustrated in table 4.

## DISCUSSION

As of today, monoclonal antibodies are the only GO-specific reagents available. Their use to study the GO has shown that different antigenic structures are present on the surface of the organism. Indeed some epitopes, those recognized by 10A6, 2D12, 12E12, 6G1 and 2H9 are of proteic nature. Among them, that recognized by 2H9 contains carbohydrates and is not transmembraneous but linked to the outer membrane by ionic strength. Thus it can be easily removed by 1M NaCl. On the contrary, 10F4, 5H10 and 11H6 are not directed against proteic epitopes. The sensitivity of the antigen to periodate suggests that hybridomas 5H10 and 11H6 are specific for carbohydrates epitopes. After all treatments, hybridoma 10F4 give a positive reaction. Whether a lipid molecule is involved in the antigen-binding site will be investigated. The use of the selected MAs to detect the GO in citrus trees from various contaminated areas in South-East Asia has revealed that in spite of their morphological similarities, different serotypes of the GO-bacterium occur in nature. Therefore, MAs seem to be too specific to develop a general method for the diagnostic of the disease. Nevertheless, their use to purify the homologous GO by immunoaffinity has been achieved (7). This opens the way for the development of other diagnostic procedures. Indeed, the DNA of the purified organisms can be extracted and cloned in order to produce DNA probes for GO-detection by DNA-DNA hybridization. This detection procedure should be less specific and probably more sensitive than that using MAs. Monoclonal antibodies are

TABLE 4
CHARACTERISTICS OF THE GREENING ORGANISM AS STUDIED WITH MONOCLONAL ANTIBODIES

| | In situ | | In plant extracts | | Purified organisms | |
|---|---|---|---|---|---|---|
| Morphology | Filamentous forms | Ovoid forms | Filamentous forms | Ovoid forms | Filamentous forms | Ovoid forms |
| Size (μm): | | | | | | |
| • length | NA[z] | NA | 1.25 to 4 | NA | 1.25 to 4 | NA |
| • diameter | 0.15 to 0.25 | 0.2 to 1 | 0.1 to 0.3 | 0.3 to 1 | 0.1 to 0.3 | 0.3 to 1 |
| Serology: | | | | | | |
| • fluorescence | + [y] in phloem | | + | + | + | + |
| • gold labeling | ND[x] | | + | + | + | + |
| • ELISA | NA | | + | | + | |

[z] NA = not aplicable
[y] + = positive reaction
[x] ND = Not done

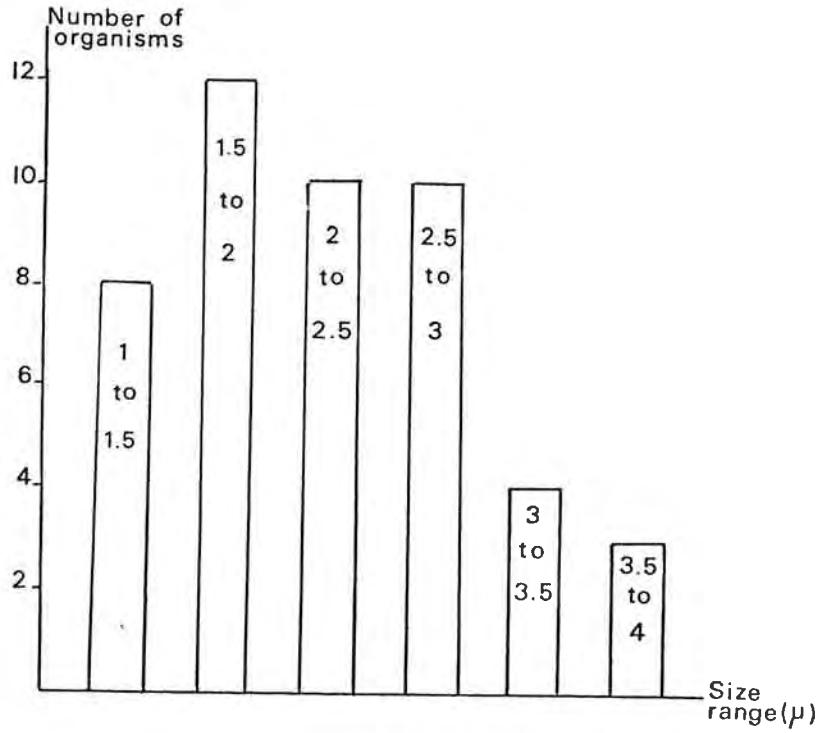


**Fig. 3. Length distribution of greening organism filaments.**

very useful tools for fundamental studies of the GO. Already, some new information on the morphology and serological characteristics of the GO have been obtained. Purification and characterization of some antigenic proteins of the outer envelop of the GO cell wall are in progress.

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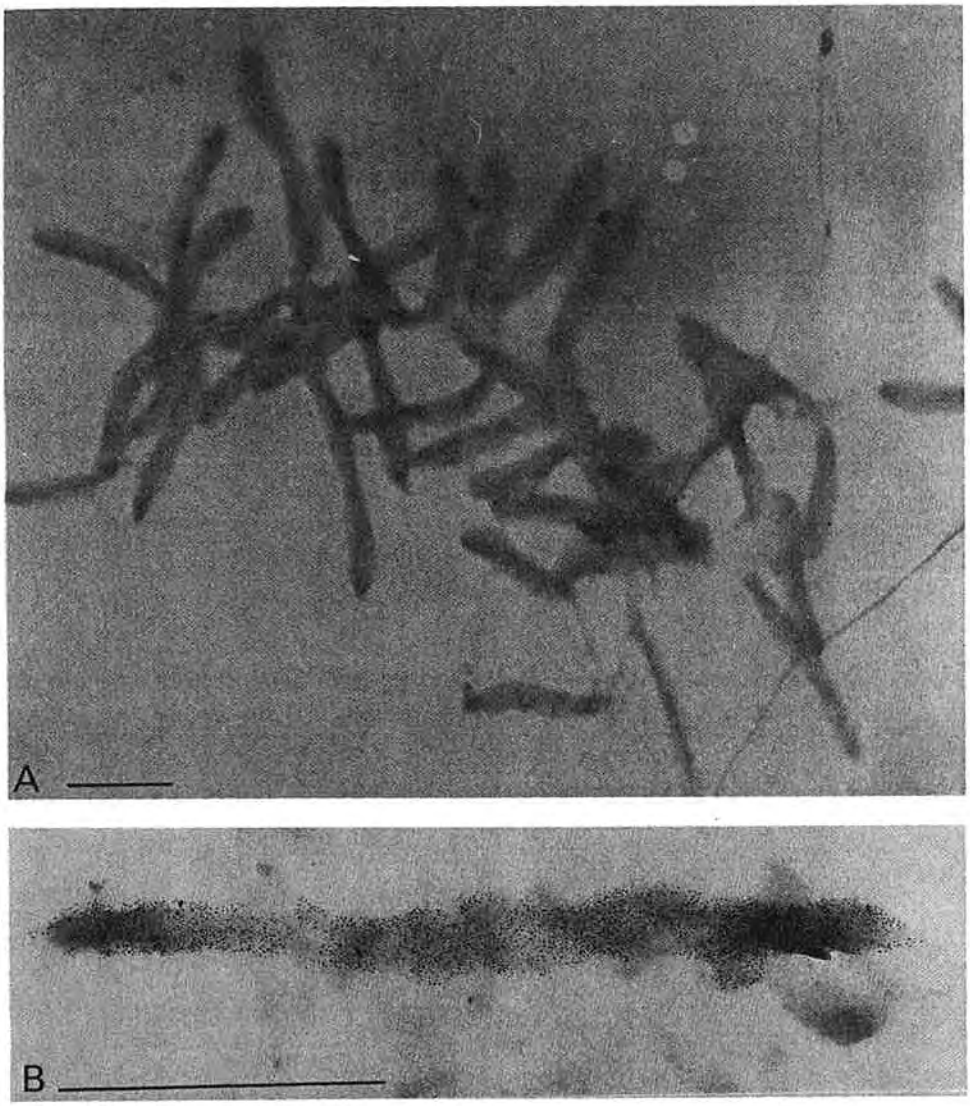


**Fig. 4. Immunoaffinity-purified Poona-GOs (A). Immunogold labeling of a purified Poona-GO with 10A6 monoclonal antibody (B). Bars = 1μm.**