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13. ABSTRACT (Maximum 200 words) Our research is based on our finding that the β_{II} isotype of tubulin is found in the nuclei of breast cancer cells. Our goals are to learn the function of nuclear β_{II} and to design a novel anticancer drug to target it. We have found that β_{II} occurs in the nuclei of human breast tumor cells <i>in situ</i> but not in normal cells. Using cultured breast cancer cells, we have found a correlation between the presence of nuclear β_{II} and the presence of estrogen receptor. We have also found that breast cancer cells which do not have nuclear β_{II} in culture, acquire it after implantation into mice. This implies that the nuclear localization of β_{II} is under hormonal control with the likely hormone being estrogen. We have also found that the distribution of nuclear β_{II} is correlated with resistance to taxotere. In another cell type, we have found that nuclear β_{II} co-localizes with vault ribonucleoprotein, which is involved in drug resistance, estrogen receptors and nucleocytoplasmic transport. We have also synthesized a peptide which rapidly enters the nucleus; we are in the process of coupling that peptide to an anti-tubulin drug to test our first antitumor drug which targets nuclear β_{II} .				
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FOREWORD

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Richard F. Ludewig

May 20, 1999

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Date

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INTRODUCTION

The research described here arises from two observations: 1) that tubulin, the subunit protein of microtubules, has been a very successful target for anti-tumor drugs (1); and 2) that the β_{II} isotype of tubulin, which normally occurs in the cytosol of most cells, is located in the nuclei of various cultured breast cancer cell lines. Microtubules are organelles that play critical roles in mitosis; their disruption can stop cell division, hence the utility of tubulin-binding drugs in cancer chemotherapy (2,3). However, virtually all normal cells also contain microtubules, thus limiting the usefulness of these anti-tubulin drugs. Normally, it appears that most cells have tubulin only in the cytosol, hence our discovery that one form of tubulin, β_{II} , is found in the nuclei of breast cancer cell lines as well as prostate and lung cancer lines is potentially of great significance. The aim of our research is two-fold: 1) to elucidate the role of the nuclear β_{II} -tubulin and 2) to design a drug which will specifically target nuclear tubulin; since nuclear tubulin is absent in most normal cells, such a drug might be specific for cancer cells. For the first aim, we are planning to microinject isotype-specific antibodies into breast cancer cells to see how their function is disrupted. As preparation for this task we have examined the distribution of the β_{II} isotype in various types of breast cancer cells and in actual tumors. We have shown that β_{II} occurs in the nuclei in actual breast tumors and that the pattern of its nuclear localization may be correlated with estrogen receptor expression and taxotere-resistance. We have also found a possible functional connection between nuclear β_{II} and the vault ribonucleoprotein. For the second aim, we have proposed three approaches to synthesizing an anti-tubulin drug which targets nuclear tubulin. We have commenced one of these approaches: we have made a nuclear targeting peptide, microinjected it into cells and shown that it readily accumulates in the nuclei. We are in the process of attaching the anti-tumor drug colchicine to this peptide.

BODY

TASK 1: MICROINJECTION OF ISOTYPE-SPECIFIC ANTIBODIES INTO BREAST CANCER AND NORMAL CELLS

Rationale:

The underlying goal of this task is to elucidate the function of nuclear tubulin. One way to approach this will be to microinject isotype-specific antibodies into normal and transformed cells to see which ones cause disruption of cellular processes. We have also found other approaches to elucidating this question. These include co-localization of nuclear β_{II} with other proteins and correlation of nuclear β_{II} occurrence with other properties of the tumor cells, including drug sensitivity. In addition, I have been concerned that the results on which the original grant proposal was based arose from experiments comparing transformed (MCF-7 and MDA) breast cancer cells with "normal" cells. It is not clear to me that there could be a completely "normal" non-transformed cell in culture so we have attempted to strengthen the basis of this research by examining breast tumors *in situ*.

Experimental Results:

Occurrence of Nuclear β_{II} in Breast Cancer Cells in Situ.

Although cultured cells are highly useful for cytological studies including immunofluorescence, it is always possible that their properties in culture may not reflect their properties in an actual tumor. In a collaborative experiment with Dr. Arlette Fellous of the Laboratoire de Pharmacologie Experimentale et Clinique in Paris, France, we have examined the occurrence of nuclear β_{II} in actual breast tumors as opposed to cultured cells. Our anti- β_{II} was applied to an excised section of a breast cancer tumor from a patient. The section was stained using the immunoperoxidase method, similar to one which we have previously used to study tubulin isotype distribution in human tissues (4). We found that β_{II} occurs in the nuclei of the breast tumors (Figure 1) and to a much lesser extent in the nuclei of the non-tumor adjacent cells (Figure 2) (See Appendices). In the breast tumor cells, the highest concentration of β_{II} appeared to be in the nucleoli (Figure 1); in fact, the pattern of β_{II} distribution in the nuclei appeared to be identical to the one we have previously observed in rat kidney mesangial cells (5). Interestingly, the pattern was much more striking than the one which we observe in the cultured MCF-7 cells, in which the distribution is fairly homogeneous in the nuclei. The non-tumor cells appear to have very little β_{II} in their nuclei (Figure 2). These results indicate that the presence of nuclear β_{II} in tumor cells occurs *in situ* as well as in culture.

There May a Correlation between Nuclear β_{II} and Estrogen Receptors in Breast Cancer Cells

Using our anti- β_{II} antibody and immunofluorescence, we have observed that breast cancer cells which are rich in estrogen receptors (MCF-7 cells) have considerable nuclear β_{II} . With breast cancer cells which lack estrogen receptors (MDA cells), the extent of nuclear β_{II} is variable.

Some preparations of MDA cells have no nuclear β_{II} ; others have slightly less than do MCF-7 cells. Again in collaboration with Dr. Fellous, we also injected MDA cells lacking nuclear β_{II} into mice. Soon the transplanted cells developed nuclear β_{II} . These results imply that the appearance of nuclear β_{II} may be under hormonal control. A likely candidate for such a hormone would be estrogen. Perhaps the transport system which brings the hormone-receptor complex into the nucleus also brings in the β_{II} -tubulin, implying a role for nuclear β_{II} in nuclear transport. Our results also imply that the presence of nuclear β_{II} may be useful for screening biopsy samples to see if the tumor is likely to respond to tamoxifen.

Distribution of β_{II} -Tubulin in and among Nuclei May Be Correlated with Resistance to Taxotere

Calc18 are a human breast cancer cell line obtained from Rhone-Poulenc-Rorer. The Calc18 cells can be either resistant or sensitive to taxotere. In another collaborative study with Dr. Fellous, both cell-types were implanted into mice and then examined for nuclear β_{II} . The results showed a difference in the distribution of nuclear β_{II} . Many (at least 80%) of the taxotere-sensitive cells contained nuclear β_{II} (Figure 3), but the staining in the nuclei was diffuse (Figure 4). In contrast, fewer (perhaps about 50%) of the taxotere-resistant cells contained nuclear β_{II} (Figure 5), but those that did have it had the more normal distribution, namely concentrated in the nucleoli with some staining elsewhere in the nucleoplasm (Figure 6).

Co-localization of Nuclear β_{II} with Vault Ribonucleoprotein

One strategy for deducing the function of nuclear β_{II} is to see with which other proteins it co-localizes. We have begun to explore this question in our other experimental system, rat kidney mesangial cells, which produce flatter cells which lend themselves better for immunofluorescence than do the cultured breast cancer cells. We have now tested two other antibodies. One was specific for a component of spliceosomes. The staining pattern did not correlate at all with nuclear β_{II} ; the antibody to spliceosomes stained the nuclei in a punctate pattern where each point was roughly the same size (not shown), very different from β_{II} which occurs in both large and small areas in the nuclei. In contrast, the antibody to the vault ribonucleoprotein stained the nuclei in a pattern indistinguishable from that of β_{II} ; in fact, the two co-localized (Figures 7 and 8). This finding raises the possibility that nuclear β_{II} may be involved in the same function as is the vault ribonucleoprotein. This is a particularly interesting finding in view of recent discoveries about vault ribonucleoprotein. The vault ribonucleoprotein is thought to be involved in drug resistance in tumor cells (6-9). It is intriguing that the vault ribonucleoprotein has the same distribution pattern in the nuclei of mesangial cells as does β_{II} in the nuclei of the taxotere-resistant breast cancer cells (Figures 6 and 7), a pattern that is distinctly different from the pattern of distribution of β_{II} in taxotere-sensitive cells (Figure 4). Another apparent function of the vault ribonucleoprotein is that it binds with estrogen receptor in MCF-7 breast cancer cells (10). Again, we have seen that MCF-7 cells are rich in nuclear β_{II} . In reality, these two functions of the vault ribonucleoprotein may be merely aspects of a more fundamental function, namely an involvement in nucleocytoplasmic transport, which has been proposed to be a basic function of vault ribonucleoprotein (11) and which may explain how the presence of vault ribonucleoprotein

is correlated both with drug resistance and the estrogen receptor. It is conceivable that for vault ribonucleoprotein to function in transport, it requires the presence of β_{II} -tubulin. Our results, therefore, suggest the feasibility of using co-localization with known proteins to elucidate the function of nuclear β_{II} in breast cancer cells.

TASK 2: SYNTHESIS AND CHARACTERIZATION OF A STEROID-COLCHICINE DERIVATIVE THAT TARGETS THE NUCLEUS AND BINDS TO TUBULIN

This task was scheduled to be performed in the first year of the grant. However, the reviewers of the original application were very skeptical of whether this was a feasible approach, hence, we concentrated on the third task, originally scheduled for the second year. However, our finding that there may be a correlation between nuclear β_{II} and estrogen receptors may cause us to revisit this question in the coming year.

TASK 3: SYNTHESIS AND CHARACTERIZATION OF A PEPTIDYL-COLCHICINE DERIVATIVE THAT TARGETS THE NUCLEUS AND BINDS TO TUBULIN

This task was originally scheduled to be performed in the second year, but we have commenced it in the first year. We synthesized the following peptide:

KRPRPCGMNK*EARKTKK

(* indicates that this lysine residue is labeled with fluorescein)

The peptide was microinjected into rat kidney mesangial cells to see if it targeted the nucleus. It is clear that after 1 hour (Figure 9), the peptide is distributed throughout the cell; after 2 hours (Figure 10) the peptide is accumulating in the nucleus; after 20 hours, most of the peptide is in the nucleus (Figure 11).

Deacetylcolchicine was derivatized with γ -maleimidobutyric acid *N*-hydroxysuccinimide ester. We are in the process of adding this compound on to the cysteine residue of the peptide to create a novel drug which should target nuclear tubulin. We will then microinject this drug into breast cancer cells.

KEY RESEARCH ACCOMPLISHMENTS

Research accomplishments are still preliminary but the following are perhaps the most promising:

- Nuclear β_{II} is present in the nuclei of human breast cancer cells *in situ* but not in the non-cancerous tissue.
- Nuclear β_{II} is more prominent in breast cancer cells with estrogen receptors.
- Breast cancer cells resistant to taxotere have a different distribution of nuclear β_{II} than do breast cancer cells sensitive to taxotere.
- Nuclear β_{II} co-localizes with vault ribonucleoprotein but not with spliceosomes. Co-localization with vault ribonucleoprotein suggests a role for nuclear β_{II} in nucleocytoplasmic transport and drug resistance.
- A peptide to which we intend to couple the anti-tubulin drug colchicine migrates into the nucleus within two hours.

REPORTABLE OUTCOMES

I will shortly be submitting a grant application to the National Institutes of Health on determining the function of nuclear tubulin in mesangial cells. Although the proposed NIH research does not overlap at all with the research in this grant, the results described in this progress report will be used in the application to the National Institutes of Health as additional justification for the hypotheses advanced in that application.

CONCLUSIONS

Our major conclusions, although still preliminary, are that the distribution of nuclear β_{II} in breast cancer cells may be correlated with the presence of estrogen receptors in those cells and also with their sensitivity or resistance to taxotere. Also, the co-localization of nuclear β_{II} with vault ribonucleoprotein suggests that nuclear β_{II} may play a role in drug resistance. In addition, we have made substantial progress toward synthesis of a peptide coupled with an anti-tubulin drug that will target the nucleus.

The most exciting implications of our findings is the possibility that the presence of nuclear β_{II} as well as its intra-nuclear distribution could be used as a pre-treatment screening to select an optimal therapeutic mode for the patient. For example, if we know, based on the nuclear β_{II} distribution, that the tumor is likely to be resistant to taxotere or tamoxifen, then we would avoid wasting the patient's time with those treatments. On the other hand, if we know that taxotere is likely to be a useful treatment, then we could start the treatment earlier when it would be more useful. In the case of tamoxifen treatment, there are already ways to measure directly the presence of estrogen receptors, and hence to predict the utility of tamoxifen treatment, however, there is as yet no quick way to predict whether a tumor would be resistant or sensitive to taxotere. It is possible that the our results could lead to the development of a screen. It may be a good idea to look at other cell lines that are resistant and sensitive to taxotere.

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APPENDICES

FIGURE LEGENDS

Figure 1. β_{II} -Tubulin in the Nuclei of Breast Cancer Cells. β_{II} -Tubulin in human breast cancer biopsy sections was detected by the immunoperoxidase method. Paraffin-embedded mammary tissue was de-paraffined by treatment with LMRsol-ethanol-water. Endogenous peroxidase activity was inhibited with H_2O_2 and non-specific sites were blocked with fetal calf serum. Slices were incubated with anti- β_{II} overnight at 4 °C and then with anti-mouse biotinylated antibody and a complex of streptavidin horseradish peroxidase. Peroxidase activity was detected using aminoethylcarbazol. Sections were counter-stained with hematoxylin, mounted, and examined with a microscope. In the immunoperoxidase method, the staining for the antigen is brown. Note the increased concentration of β_{II} in the nuclei and particularly in the nucleoli. The arrow points to a region where many nuclei containing β_{II} are together. The staining for β_{II} in the nuclei is sufficiently strong to overwhelm the counter-stain which should otherwise be staining the nuclei blue-green.

Figure 2. Lack of Nuclear β_{II} -Tubulin in Cells Adjacent to the Tumor Cells. Cells from a biopsy were fixed and stained with anti- β_{II} using the immunoperoxidase method, as in Figure 1. Notice that there is very little β_{II} in the nuclei, which appear blue-green. (arrow).

Figure 3. Nuclear β_{II} is Very Common in Taxotere-sensitive Breast Cancer Cells. Taxotere-sensitive Calc18 cells were implanted into mice and the resulting tumors were excised, sectioned and stained for β_{II} as in Figure 1. In this low magnification micrograph, note that most cells have nuclear β_{II} .

Figure 4. Intra-Nuclear β_{II} Distribution is More Diffuse in Taxotere-Sensitive Cells. Cells prepared as in Figure 3 are viewed at high magnification. Note that the distribution of β_{II} in those nuclei that contain it is fairly homogeneous with just a slightly higher intensity in the nucleoli.

Figure 5. Distribution of Nuclear β_{II} in Taxotere-resistant Breast Cancer Cells. Taxotere-resistant Calc18 cells were implanted into mice and allowed to develop into tumors. Sections were prepared and stained for β_{II} as in Figure 3. Comparing Figure 5 with Figure 3, the nuclear β_{II} in Figure 5 is somewhat less widespread but appears to be more intense in those nuclei that contain it.

Figure 6. Nuclear β_{II} is Less Evenly Distributed in Taxotere-resistant Cells. Taxotere-resistant Calc18 cells were stained as in Figure 5. Comparing Figure 6 with Figure 4, note that several cells in Figure 6 have little or no nuclear β_{II} , but, in those that do have it, the staining is both more intense and more concentrated in the nucleoli than is the case in Figure 4.

Figure 7. Distribution of Vault Ribonucleoprotein in Mesangial Cells. Cultured rat kidney mesangial cells were stained with an anti-vault antibody and then with secondary antibody coupled to rhodamine. Note the high concentration of vault in the nuclei and particularly in the nucleoli.

Figure 8. Co-Localization of β_{II} and Vault Ribonucleoprotein in Mesangial Cells. The same slide from Figure 7 was stained with anti- β_{II} and then with secondary antibody coupled to fluorescein. Note that the distribution of β_{II} is essentially indistinguishable from that of the vault ribonucleoprotein in Figure 7.

Figure 9. Peptide with Nuclear Localization Sequence One Hour after Microinjection into Mesangial Cells. The peptide KRPRPCGMNK*EARKTKK (the * indicates a lysine labeled with fluorescein) was microinjected into rat kidney mesangial cells and viewed after one hour. Note that the label is widespread throughout the cytoplasm of injected cells.

Figure 10. Peptide with Nuclear Localization Sequence Two Hours after Microinjection into Mesangial Cells. Microinjected cells were viewed after two hours. Note that the label is largely concentrated in the nuclei.

Figure 11. Peptide with Nuclear Localization Sequence Twenty Hours after Microinjection into Mesangial Cells. Microinjected cells were viewed after 20 hours. Note that the label is still largely concentrated in the nuclei.



Figure 1



Figure 2

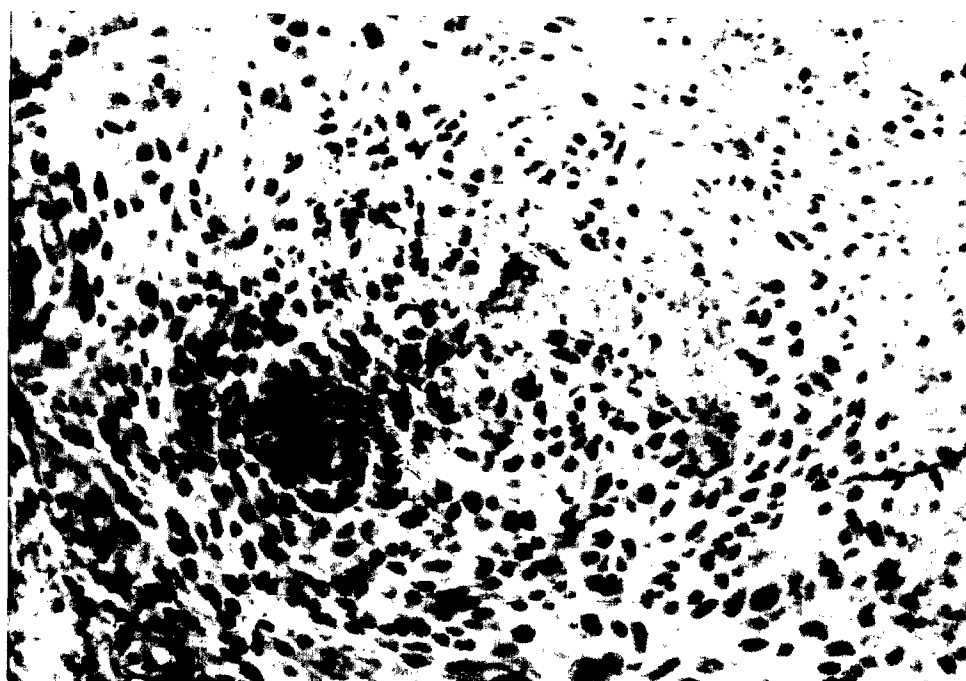


Figure 3

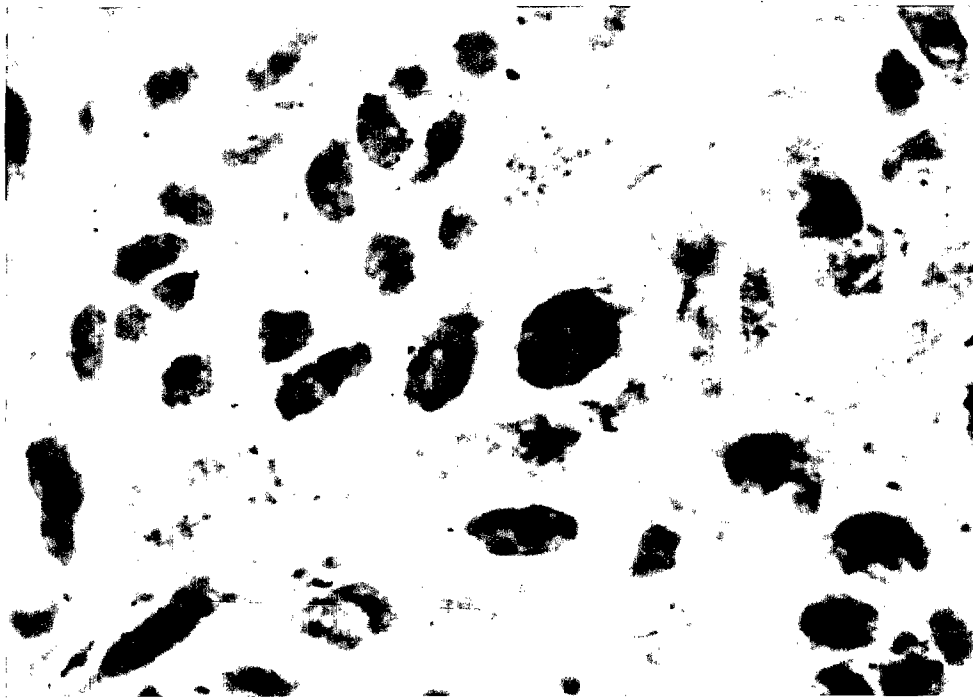


Figure 4

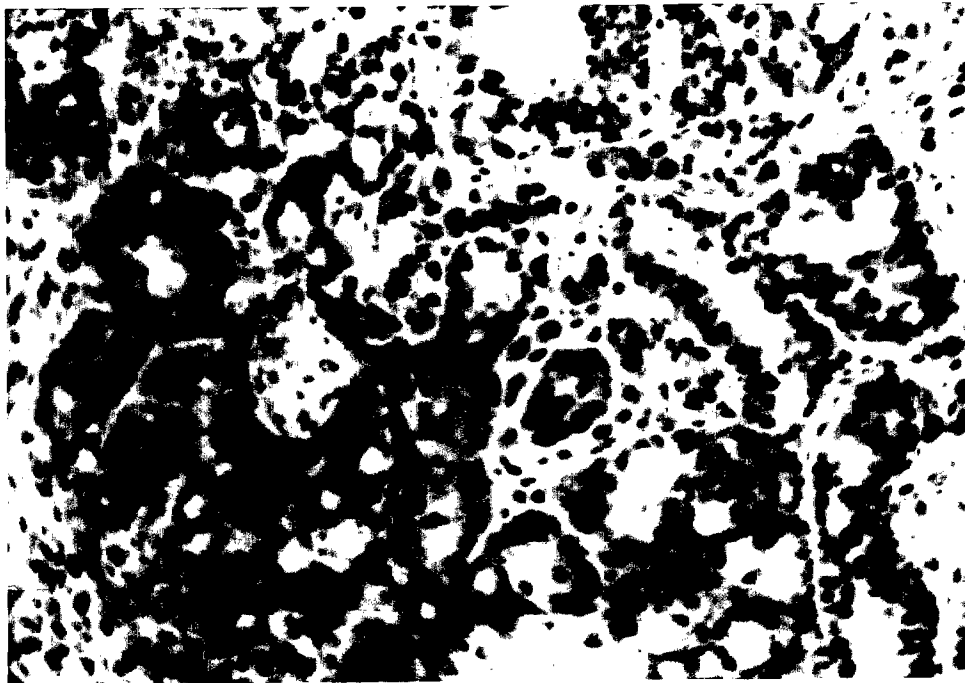


Figure 5

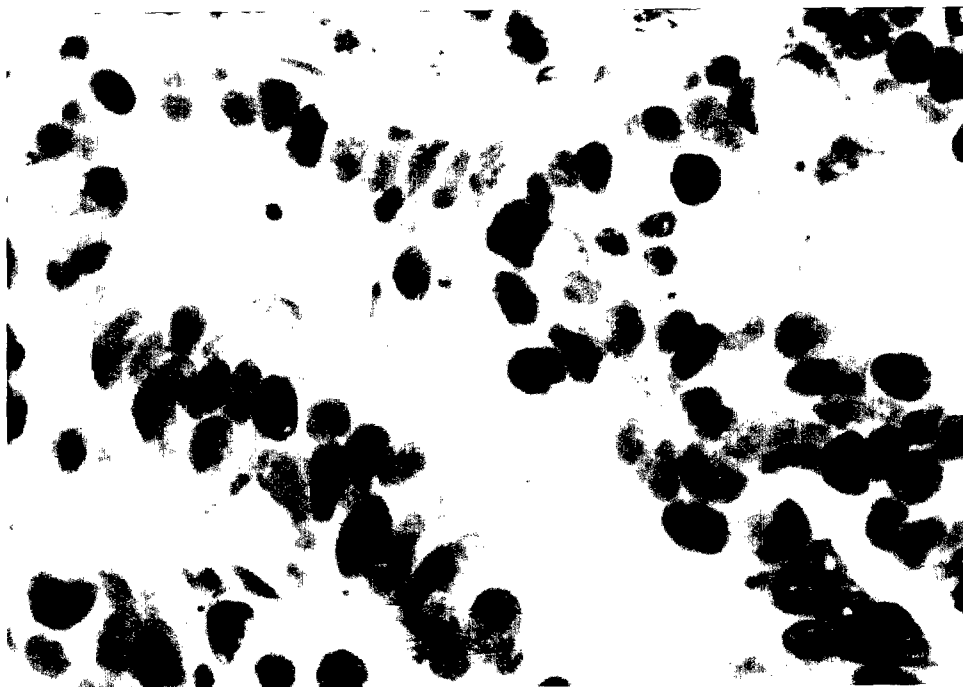


Figure 6



Figure 7

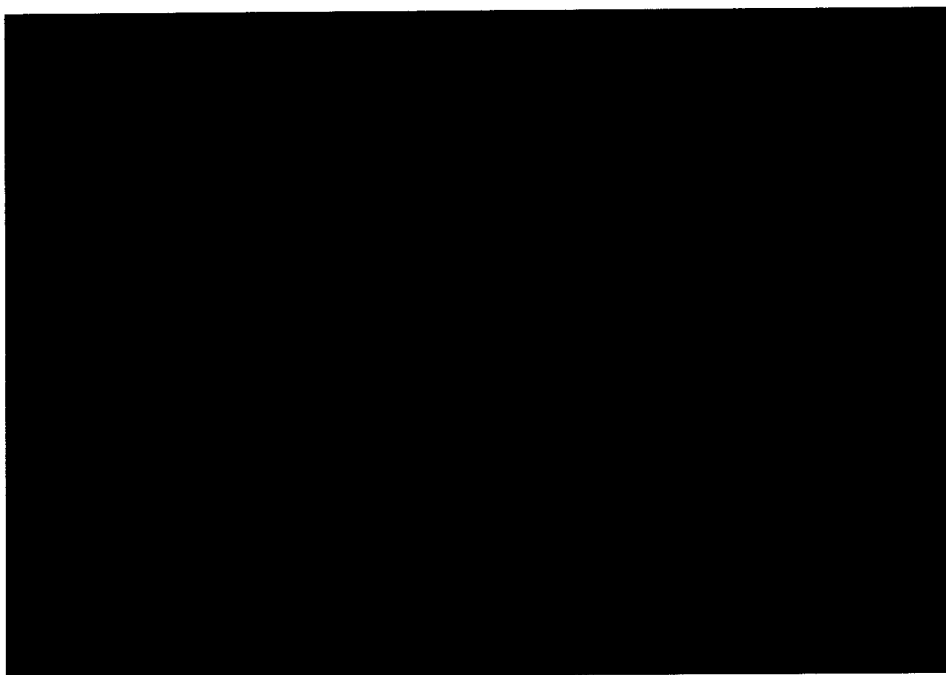


Figure 8



Figure 9

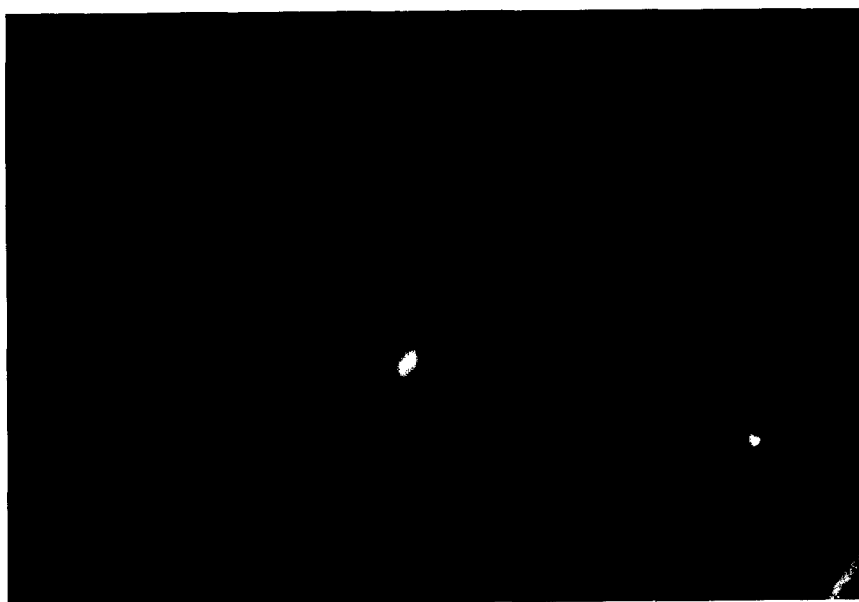


Figure 10

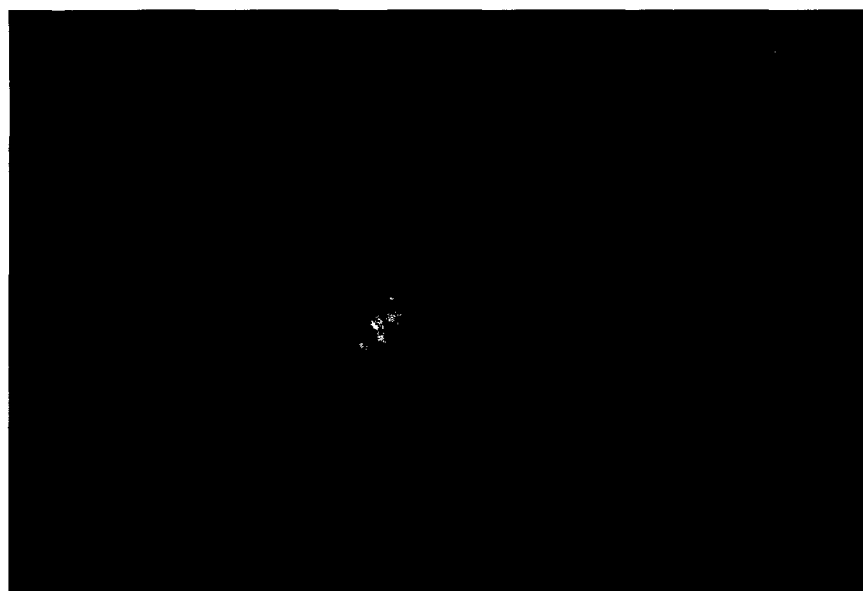


Figure 11



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