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Breast cancer is the second most common cause of cancer mortality among women in the United States, and as such it is a disease with substantial clinical relevance. Several lines of evidence, including both epidemiological studies in humans and carcinogen studies in rodents, have suggested that puberty represents a period of enhanced susceptibility to carcinogenesis in the breast. We hypothesize that a change in the epithelial cell subtypes present during puberty accounts for the altered susceptibility to carcinogenic insult. We have investigated epithelial lineage in the developing murine breast by using retrovirally tagged mammary epithelial cells to repopulate mammary gland fat pads. We have also generated noncrosshybridizing pools of cDNA which are characteristic of pubertal versus adult mammary gland in order to identify genes which can function as markers for epithelial cell subtypes characteristic of puberty. Investigation of the relationship between puberty, differentiation, and breast cancer risk may yield fundamental insights into mechanisms of carcinogenesis.

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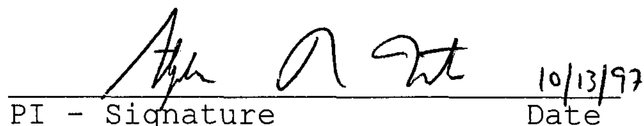

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Introduction:

Puberty and the Risk of Breast Cancer

There have been at least three independent lines of epidemiological evidence suggesting that the breast is susceptible to carcinogenic insults such as ionizing radiation during puberty. First, a long-term followup of women who survived the atomic bomb blasts at Hiroshima or Nagasaki reported that the greatest risk of subsequent breast cancer was found in women who were between the ages of 10 and 19 at the time of irradiation [1]. Second, women who were repeatedly examined by fluoroscopy during treatment for tuberculosis have also been found to have an increased risk of developing breast cancer [2]. Once again, the greatest excess in breast cancer occurred in individuals whose first X-ray exposure occurred between ages 15 and 19. Finally, women treated with mantle-field irradiation for Hodgkin's disease before the age of 20 have been found to have a significantly higher risk of developing breast cancer than women treated at ages 20-29 [3]. While in most cases a substantial period of time elapsed between the initial mutagenic insult and the clinical appearance of breast cancer, it is clear from these studies that during puberty (at least compared to early adulthood) the breast is especially vulnerable to early steps in carcinogenesis. Understanding the molecular underpinnings of breast development and differentiation during puberty, therefore, should yield fundamental insights into the series of events which can lead to breast cancer later in life.

This hypothesis is further supported by an animal model in which virgin rats are fed the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) and develop mammary gland carcinomas [4]. As in humans, rats in this model are most susceptible to develop breast carcinoma if exposed to carcinogen during puberty. Of particular interest in these studies was the observation that the risk of developing breast cancer upon treatment with DMBA was directly correlated with the number of undifferentiated structures, known as terminal end buds, present in the breast. Moreover, in this model system, DMBA-induced carcinomas appear to arise from the terminal end buds themselves in conjunction with their aberrant development [5]. Thus, these studies also suggest that structures and pathways of differentiation which are associated with puberty may play a fundamental role in the development of breast cancer.

Terminal End Buds

In addition to their strong correlation with susceptibility to carcinogenic insult, terminal end buds are of considerable interest for understanding the basic mechanisms of breast development and branching morphogenesis (for review, see [6]). Terminal end buds appear at the tips of the primitive mammary ductal structure in the FVB mouse at approx. 3 weeks of age, and they rapidly grow and branch over the next 4-6 weeks as they give rise to the epithelial tree that fills the mammary fat pad. When these highly proliferative structures reach the end of the mammary fat pad, they regress and the rapid development which is characteristic of puberty ends.

Morphologically, terminal end buds are composed of a single layer of highly proliferative "cap cells" found at the tip of the bud, underneath which are found 3-5 additional layers of cuboidal epithelium which are known as "body cells". Cap cells are anatomically continuous with the myoepithelial layer, which in conjunction with a gradient in marker expression provides some evidence that cap cells give rise to myoepithelia. Body cells are in turn thought to give rise to the luminal epithelium. In addition, microcinematography of a terminal end bud suggests that cap cells may give rise to body cells [7], and thus cap cells may function as progenitor cells which give rise to all other mammary epithelial cell subtypes.

This interpretation of terminal end bud lineage is supported by the groupings of immunohistochemical marker expression in various mammary cell types [8-10]. In particular, monoclonal antibodies which can distinguish luminal epithelium from

myoepithelium are both expressed in cap cells; from this data it is inferred that cap cells represent a progenitor cell population which can give rise to both differentiated cell types. Furthermore, these antibodies (which in one case recognize a specific set of cytokeratins and in the other case recognize an unknown cell-surface antigen) colocalize to more primitive cells seen early in breast development and to isolated myoepithelial cells in older glands. Additional studies suggest that such a "basal cell" population expresses a separate set of antibody-detected glycoproteins characteristic of cap cells but not of most luminal or myoepithelial cells [11, 12]. It should be noted, however, that these markers were also detected in a small subset of luminal cells; thus it is not clear whether they are entirely specific.

While the use of monoclonal antibodies as immunohistochemical markers has been helpful in generating basic hypotheses, these studies have several limitations. First, they rely on similarities in marker expression to infer lineage relationships between cell types. While parsimonious marker clusterings are useful and suggestive, a rigorous establishment of lineage depends (short of direct *in vivo* observation) on using a heritable marker to identify the progeny of a given cell. Second, they limit the amount of further information which may be obtained about the nature of the marker; it is fairly difficult to characterize the epitope which is recognized by an anonymous monoclonal antibody. Finally, it is difficult to elucidate the mechanism of regulation of a marker detected via immunohistochemistry. A change in the distribution of epitopes may reflect transcriptional regulation, post-transcriptional alteration of mRNA stability, or some form of post-translational processing via the masking or alteration of an epitope.

Additional Epithelial Cell Subtypes

Considering the evident importance of defining both the lineage and phenotype of epithelial cell subtypes during mammary development, it is remarkable that relatively few markers for such cell subtypes exist (see [13] for review) in addition to the markers already mentioned in the discussion of terminal end buds. The presence of a rich set of markers is extremely important for the study of breast cancer in that the increased susceptibility to breast cancer seen during puberty may be due either to global (e.g. hormonal) influences on all cell types or to the particular collection and relative abundances of epithelial cell types during puberty as compared with adulthood. A major goal of this project, therefore, has been to identify genes which are differentially regulated in various epithelial subtypes. That is, any gene which is expressed in only a subset of cells may be used as a marker to "define" that subtype of epithelial cell. Once subtypes are defined, their location and relative abundance can be followed throughout mammary development. It should be noted that the actual function of these genes does not need to be determined in order for them to serve as useful markers for epithelial cell subtypes. If the more abundant presence of a given cellular subtype is responsible for the increased risk seen during puberty, it may be possible to use such markers to determine the relative susceptibility to carcinogenic injury in a given sample of breast tissue; clearly such markers would have substantial clinical importance [14]. It is clear, therefore, that the generation of such markers would substantially facilitate the understanding of both breast development during puberty and, consequently, of the alterations in breast cancer risk which occur as a result of puberty .

Lineage studies

While the studies of Daniel, Dulbecco and others have inferred lineage relationships on the basis of anatomy and distribution of shared markers, this evidence is indirect in nature. Indeed, in order to rigorously identify the progeny of any given cell it is necessary to "tag" that cell with a heritable marker. As initially shown in the retina, this may be accomplished with the use of replication-defective retroviruses encoding histochemical markers such as b-galactosidase or human placental alkaline phosphatase [15-17]. Infecting retinal cells with these retroviral vectors causes the integration of the histochemical marker into the genome of a dividing, infected cell. Thus, all progeny of the

infected cell will also express the histochemical marker and can be identifying by staining with the appropriate substrate. By ensuring that only a single clone was being analyzed at a given time, Cepko and colleagues were able to demonstrate the range of retinal cell types that can arise from a single precursor cell. Such work demonstrated the somewhat surprising fact that a common progenitor cell can give rise to most cell types in the retina, including both neurons and glia [18, 19]. A similar study in the breast aimed at demonstrating the presence of one or more progenitor cell types within the mammary epithelium would be of significant value since these relatively undifferentiated cells may be targets for carcinogenesis.

Specific aims:

To address the issues raised in this introduction, we proposed three specific aims:

1) ***Identify and Localize Progenitor Cells in the Pubertal Breast***

2) ***Identify changes in mammary epithelial cell subtypes occurring during puberty***

3) ***Delete epithelial cell subtypes and study the effect on development and risk of breast cancer***

Body:

Technical Objective 1: Identify and Localize Progenitor Cells in the Pubertal Breast

Task 1: Months 1-5: Infection of terminal end buds with recombinant retrovirus

In order to directly address the question of epithelial lineage in the developing breast, we proposed direct infection of terminal end buds with a replication-defective recombinant retrovirus--visualization of these end buds would be facilitated by IP injection of trypan blue into pubertal mice followed by surgery with visualization of TEBs under a dissecting scope the next day. Our attempts to visualize the TEBs in this way, however, were less successful than we anticipated. Not only did it prove very difficult to see terminal end buds with any degree of certainty, but the consistency of the gland itself made injection of virus into such a small, defined area quite difficult. Presumably it is not enough to merely inject the virus in the proximity of the TEB, since a basement membrane separates the fat pad and mesenchyme from the terminal end bud epithelium. In order to get around this problem, we made several attempts to inject directly into the #4 nipple of an anaesthetized mouse. While this technique has been successful in a rat model [20], in our hands it was not possible to reproducably canulate the nipple of an FVB mouse. Thus, we conclude that at this time technical problems make it impossible for us to adequately perform this type of experimental analysis.

Task 2: Months 1-9: Use of histochemically tagged primary MEC to reconstitute a cleared breast

We hypothesized that undifferentiated progenitor cells, which may be susceptible targets for carcinogenesis during puberty, can give rise to both luminal and myoepithelial lineages in the pubertal breast. To address this question we attempted to use histochemically tagged

primary mammary epithelial cells (MECs) to reconstitute a cleared breast. This technique allowed us to specifically define the number of progenitor cells contributing to the formation of the reconstituted mammary epithelial tree. Primary MECs were harvested from pubertal (5-wk) FVB mice by treatment with collagenase followed either by centrifugation through a Percoll gradient or by allowing the epithelial tree to settle out of, e.g., a 10 mL suspension over a period of 10 minutes. This cell population, which is highly enriched for primary MECs, was then plated onto a feeder layer of mitomycin C-treated retroviral producer cells. These cells produce replication-incompetent retroviruses which express either beta-galactosidase or alkaline phosphatase; culturing primary cells on this layer thus allowed us to infect at reasonable efficiency. Previous work by both our lab and others (unpublished data, and [21]) had suggested that an entire branch of epithelial tree might arise clonally from a single precursor; however, it has also been suggested that in certain cases a group of several cells may give rise to such a branch. Since it has been shown that spontaneous inactivation of a transgene tag can occur (thus not allowing nonstaining cells to be scored for the purpose of lineage analysis), the only way to demonstrate this conclusively, however, is to use more than one marker; in this way a branch of epithelial tree derived from more than a single precursor can be unambiguously scored.

After primary MECs had been harvested and retrovirally tagged, they were injected into the #4 mammary glands of 3-week-old mice from which all mammary gland tissue proximal to the lymph node (contained in the gland) had been removed. Thus, all endogenous mammary epithelium had been removed from the #4 gland prior to injection of cultured MECs. ~8-12 weeks after injection, the mice were sacrificed and the mammary glands were removed. After fixation of whole-mount glands in 0.5% gluteraldehyde, they were stained with X-gal for beta-galactosidase expression. After sufficient staining time, the gland was heated to 65°C in order to inactivate endogenous (heat-sensitive) alkaline phosphatase prior to staining for exogenous (heat-resistant) alkaline phosphatase expression. After post-fixing, glands were defatted using toluene and methyl salicylate (glands were backed out into PBS for longer-term storage). After visualization of the whole-mount, some glands were embedded in paraffin for sectioning.

While outgrowths differed in the extent of their repopulation of the cleared fat pad, is was nonetheless possible to detect beta-galactosidase and alkaline phosphatase activity both in whole mounts and (to a lesser extent) in sections. While it has been possible in the past to generate very clean results using a single tag, however, we found that technical problems have confounded our attempts to use both beta-galactosidase and alkaline phosphatase staining in this context. Specifically, heat-inactivation of endogenous alkaline phosphatase appears to adversely affect the quality of the overall results obtained in the context of a whole-mount gland. Further, sufficient staining which will not leach out during dehydration and defatting of the gland in organic solvents also appears to yield some nonspecific background staining around terminal end buds. While at first glance it might seem possible to avoid potential diffusion problems by simply staining after embedding and sectioning the gland, this technique would bring with it the need for extensive digitizing of sections along with three-dimensional reconstruction of the morphology of the tree using thin serial sections. This obviously adds a significant degree of difficulty to the completion of this type of analysis. Furthermore, since University regulations require that mice injected with cells containing a replication-deficient retrovirus be housed under Biosafety Level II conditions (over the course of several months using this protocol), there is a significant cost associated with these experiments; as this training grant included no supply budget, this cost becomes an issue in continuing these investigations. For these reasons (technical and financial), then, we are not continuing our experiments on this arm of the project.

Technical Objective 2: Identify changes in mammary epithelial cell subtypes occurring during puberty.

We hypothesized that changes in the number and distribution of epithelial cell subtypes might account for the increased risk of carcinogenic injury during puberty. We therefore attempted to identify cDNA markers which could define subtypes of mammary epithelial cells in order to follow the changes in abundance of these cell types during puberty. We attempted to identify particular messages which were up- or down-regulated in 5-week (pubertal) versus 15-week (adult) FVB mice, hypothesizing that these messages would be most likely to define distributions of epithelial cell subtypes which characterize the pubertal breast.

Task 1: Months 1-9: Select candidate markers

1. Differential screening of cDNA libraries

We constructed cDNA libraries using poly(A)+ RNA isolated from 5- and 15-week virgin mouse breast tissue. Each of these libraries was seperately plated, and duplicate filter lifts were prepared. To isolate genes that are expressed in mid-puberty (5 weeks) and which are not expressed later in development (15 weeks), filters from the 5-week virgin library were probed with 32P-labeled single-stranded cDNA prepared from either 5-week or 15-week virgin mammary gland poly(A)+ RNA. Similarly, filter lifts representing the 15-week virgin mouse mammary gland library were probed with labeled single-stranded cDNA derived from either 5- or 15-week mouse mammary gland RNA. In each case, plaques which hybridized to "self" probe but not to "nonself" probe should represent genes that are differentially regulated during development. $>10^5$ clones were screened from the 5-week library, and ~3000 clones were screened from the 15-week library. Secondary screens were performed for 13 potentially positive clones from the 5-week library and 6 clones from the 15-week library. In both cases no clones were found to be differentially expressed after the secondary screen. While this technique had previously been used in our laboratory to isolate genes which are specifically expressed by a subset of cells in parous mammary epithelium, we realized that this technique requires that a gene be present at a sufficiently high copy number to effectively hybridize. This, then, may provide a potential explanation for the failure of our screen to detect genes that are differentially expressed between pubertal and adult breast.

2. PCR-based subtractive hybridization

We used the method of Wang and Brown ([22, 23]) to identify differentially regulated genes that were expressed at low levels. Briefly, poly(A)+ RNA from 5- and 15-week virgin female FVB mice was used to generate double-stranded cDNA. Separate aliquots of each cDNA pool were digenested with *Alu*I or *AluI/Rsa*I and ligated to a double-stranded linker. Linker-ligated cDNA was amplified using PCR with primers complementary to the linker region. 15-week amplified cDNA was photobiotinylated and incubated with 5-week amplified cDNA. After boiling, the mixture was allowed to hybridize at 68°C for 20 hrs. Straptavadin was used to remove strands which hybridized to the biotinylated cDNA, yielding a pool of cDNAs which are enriched for messages which are differentially expressed. A second, short hybridization was then used to aid in the subtraction of abundantly expressed messages. This procedure was used to subtract 15-week from 5-week cDNA as well as 5-week from 15-week. Enriched pools were then reamplified for use as both driver and tracer, respectively, in further rounds of subtraction.

We initially performed 6 rounds of subtraction (3 long and 3 short hybridizations) as had Wang and Brown, but discovered that we had not entirely succeeded in creating

non-crosshybridizing pools of cDNAs (data not shown). We therefore performed an additional 2 rounds of subtraction, and the resulting cDNA populations do not cross-hybridize on Southern blot (Figure 1, below).

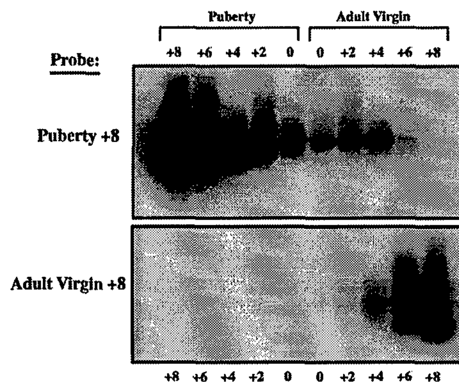

Figure 1: Southern Blot demonstrating extent of pubertal and adult cDNA cross-hybridization after 8 rounds of subtraction. Successive rounds of enrichment are labelled (e.g. +2, +4, etc.). Note particularly the absence of detectable hybridization between labelled "Puberty+8" probe and cDNA in the "Adult Virgin+8" lane as well as between labelled "Adult Virgin+8" probe and cDNA in the "Puberty+8" lane.

Task 2: Months 10-12: Characterize candidate marker genes

To determine if cDNAs generated by this procedure truly exhibited differential expression in the 5- vs. 15-week virgin FVB mammary gland, we subcloned fragments into Bluescript II and generated probes for use in Northern hybridization. While it was possible to demonstrate that some genes were up- or down-regulated in the pubertal breast, in no case did we see a gene with a >3-fold change in expression. Furthermore, >50% of fragments did not yield a detectable signal with Northern hybridization. In order to get a better idea of whether these cDNAs reflected known gene families, a number were sequenced and used to search GenBank; the vast majority did not show any homology to known genes. On the one hand, it is exciting to have identified a set of novel genes with potential biological relevance; on the other hand, the difficulty of detecting most of these genes, coupled with their lack of homology to known genes, makes it difficult to predict the utility of these genes as markers. Mere ascertainment of whether they are differentially regulated will require generating probes for RNase protection assays along with optimizing the conditions for such an assay on a probe-by-probe basis. Furthermore, while genes with levels that can be assessed via Northern are encouraging to the extent that they tend to show modest levels of differential expression during puberty, it is far from clear whether such a mild change (<3-fold upregulation) is likely to reflect a major shift in epithelial cell subtypes. In both of these cases (undetectable, or detectable with mild change) the only way to screen for an interesting expression pattern is via *in situ* hybridization.

Technical Objective 3: Delete epithelial cell sutbypes and study the effect on development and risk of breast cancer

Task 1: Months 12-24: Isolate promoters which are regulated in an epithelial cell subtype-specific fashion

Work on this portion of the project has not commenced, since it first requires results from the *in situ* hybridization screen alluded to in technical objective 2, task 2.

Task 2: Months 25-36: Delete epithelial cell subtypes in a transgenic mouse

Work on this portion of the project has not commenced, since it requires completion of task 1.

Task 3: Months 36-48: Determine the susceptibility to breast cancer of transgenic mice in which specific epithelial cell subtypes have been ablated

Work on this portion of the project has not commenced, since it requires completion of task 2.

Summary/Additional data/Plans for continued work:

The purpose of this grant has been to address fundamental issues relating to the relationship between development, differentiation, and breast cancer risk. To this end we have attempted both lineage analysis in the murine breast and isolation of differentially regulated messages which reflect shifting populations of epithelial cell subtypes. As discussed under Technical Objective 1, lineage studies have met with certain technical challenges; furthermore, since the training grant includes no supply budget, fiscal issues have limited the additional work which would be required to troubleshoot this experimental system. As discussed under Technical Objective 2, multiple techniques have been attempted in order to isolate genes that are differentially regulated during puberty. Since these techniques have not yielded genes with >3-fold upregulation, it seems evident to us that in the absence of strong candidates we will be required to screen a large number of genes from our enriched pools via *in situ* hybridization in order to determine their biological interest as markers for puberty-specific (or -predominant) epithelial cell subtypes. This also would be an extremely costly and lengthy endeavor, and the absence of supply budget once again limits our ability to do these experiments.

In light of these considerations we have decided to adopt an alternate strategy to address the same fundamental issues. Rather than searching for genes which mark underlying processes during differentiation and development, we have decided to focus on BRCA2, a gene which has been demonstrated to have a profound role in a large number of familial breast cancers ([24, 25]). BRCA2 is of particular interest in the context of this grant in that it has been shown to be upregulated in proliferating and differentiating cells ([26, 27]) and is upregulated during puberty; furthermore, expression has been detected in terminal end buds. An understanding of the function of BRCA2, then, might reasonably be expected to yield insights into the relationship between differentiation and breast cancer risk which this grant was originally designed to investigate.

To begin our studies of BRCA2, the lab has generated a full-length human clone as well as several mutants which have a demonstrated relationship to breast cancer (999del5 and 6174delT). We have further modified this clone by introducing silent mutations which will allow us to more easily manipulate this large (>10.5 kb) gene. We have cloned this gene into both constitutive and tetracycline-inducible expression vectors, and to facilitate detection we have created full-length BRCA2 constructs which contain one of several epitope tags (HA, FLAG, with myc to follow) at the N terminus. We have demonstrated that a full-length FLAG-tagged construct is detectable on Western Blot and comigrates with endogenous BRCA2. We are in the process of creating stable cell lines which will express rtTA (the reverse tetracycline transactivator) and a tetracycline-inducible BRCA2 construct

in order to study the relationship between BRCA2 and differentiation in cell culture. Further, we have created a number of cell lines which contain a Brca2 antisense construct under control of a tetracycline-inducible promoter. These studies are ongoing, and results will be discussed in next year's annual report.

CONCLUSIONS:

Since the time this grant was submitted, we have made significant progress on a number of the proposed experimental fronts, although certain technical difficulties have hampered our ability to carry these experiments forward to a publishable conclusion at this point. First, we have been able to tag primary mammary epithelial cells in a reproducible manner using culture on feeder layers of retroviral producer cell lines. In contrast with previous work which used a single marker, we have used two markers; this will, in theory, allow the unambiguous inference of lineage. We have further been able to reproducibly repopulate cleared mammary fat pads using these cells. Unfortunately, we have encountered difficulties with staining and detection of tagged cells in whole-mounted glands, and the cost of animal housing associated with these experiments makes continued work unfeasable in the absence of a supply budget associated with this grant. We also successfully generated non-crosshybridizing pools of cDNAs from pubertal and adult FVB mice, and our characterization of these pools indicates that they do indeed contain differentially regulated genes. The modest extent of this regulation, however, leaves some doubt as to the extent of the biological relevance of this regulation, and addressing this issue would require a large-scale screen via *in situ* hybridization. Therefore, in order to address the fundamental questions proposed by our grant while avoiding the technical hurdles encountered to date, we have initiated studies of the breast cancer tumor suppressor gene BRCA2. BRCA2 has been shown to be upregulated during puberty, is expressed in epithelial structures characteristic of puberty, and is expressed in proliferating and differentiating tissues; as such it represent an excellent candidate for study under the overarching aims of this grant. To date we have obtained a full-length human clone along with several disease-causing mutations. We have created vectors for expression of these full-length constructs under the control of either constitutive or tetracycline-inducible promoters. In addition, we have created epitope-tagged versions of the protein in order to facilitate detection and manipulation of the overexpressed protein. Finally, we have obtained a number of clones which contain an antisense construct which we anticipate may allow us to study the effects of downregulating Brca2 expression on proliferation and differentiation in cell culture. We believe that these studies will allow us to make progress in understanding the fundamental relationship between development, differentiation, and breast cancer.

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DEPARTMENT OF THE ARMY
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MCMR-RMI-S (70-1y) 23 Aug 01

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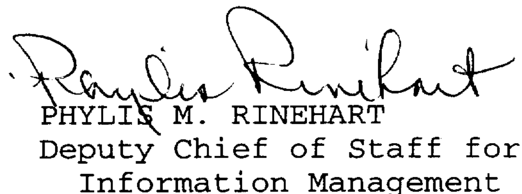

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