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ABSTRACT

The exposure of the body to microgravity during space flight causes a series of well-documented changes in Ca^{2+} metabolism, yet the cellular/molecular mechanisms leading to these changes are poorly understood. There is some evidence for microgravity-induced alterations in the vitamin D endocrine system, which is known to be primarily involved in the regulation of Ca^{2+} metabolism. Vitamin D-dependent Ca^{2+} binding proteins, or calbindins, are believed to have a significant role in maintaining cellular Ca^{2+} homeostasis.

We used immunocytochemical, biochemical and molecular approaches to analyze the expression of calbindin-D_{28k} and calbindin-D_{9k} in kidneys and intestine of rats flown for 9 days aboard the Spacelab 3 mission. The effects of microgravity on calbindins in rats in space *vs.* "grounded" animals (synchronous Animal Enclosure Module controls and tail suspension controls) were compared. Exposure to microgravity resulted in a significant decrease in calbindin-D_{28k} content in kidneys and calbindin-D_{9k} in the intestine of flight and suspended animals, as measured by enzyme-linked immunosorbent assay (ELISA). Immunocytochemistry (ICC) in combination with quantitative computer image analysis was used to measure *in situ* the expression of calbindins in kidneys and intestine, and insulin in pancreas. There was a large decrease in the distal tubular cell-associated calbindin-D_{28k} and absorptive cell-associated calbindin-D_{9k} immunoreactivity in the space and suspension kidneys and intestine, as compared with matched ground controls. No consistent differences in pancreatic insulin immunoreactivity between space, suspension and ground controls was observed. There were significant correlations between results by quantitative ICC and ELISA. Western blot analysis showed no consistent changes in the low levels of intestinal and renal vitamin D receptors.

These findings suggest that a decreased expression of calbindins after a short-term exposure to microgravity and modelled weightlessness, may affect cellular Ca^{2+} homeostasis and contribute to Ca^{2+} and bone metabolism disorders induced by space flight.

INTRODUCTION

Vitamin D is the precursor of the steroid hormone, 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] (Norman et al., 1982; Henry and Norman, 1984; Sergeev, 1989). 1,25$(OH)_2D_3$ produces a wide spectrum of biological effects via both receptor mediated regulation of nuclear events (Minghetti and Norman, 1988; Lowe et al., 1992) and rapid actions independent of the genomic pathway (Norman et al., 1992; Farach-Carson et al., 1991; Sergeev and Rhoten, 1995). The vitamin D receptor (VDR) regulates genes associated with Ca^{2+} homeostasis (*e.g.*, calbindins; Christakos et al., 1984), with the proliferation pathway, the differentiation pathway and the developmental cascade (Lowe et al., 1992). Calbindins are believed to be essential, as intracellular Ca^{2+} sequestrants/buffers, to the process of intestinal Ca^{2+} absorption (Nemere et al., 1991; Norman et al., 1992) and renal Ca^{2+} reabsorption (Johnson and Kumar, 1994; Hemmingsen et al.,1995). Calbindin-D_{28k}, functioning as an intracellular Ca^{2+} buffer, is crucial for preventing accumulation of excessive levels of cytosolic free Ca^{2+} (Iacopino et al., 1992; Rhoten and Sergeev, 1994) and, thus, determining cell fate (Dowd, 1995).

There is the evidence that the integrated operation of the vitamin D endocrine system is affected by factors of space flight, including evidence from human and rat models of microgravity (Arnaud et al., 1991; Morey-Holton et al., 1988; Sergeev et al., 1982-1986; Spirichev and Sergeev, 1988). Serum 1,25$(OH)_2D_3$ concentration decreased in healthy volunteers after one year bed rest with the head lower than the feet (Sergeev and Morukov, unpublished observations). The 1,25$(OH)_2D_3$ production in the kidney and accumulation of the hormone in the bone and intestine markedly decreased in rats after long-term hypokinesia (Sergeev et al., 1983; 1984). These were accompanied by a decrease in the intestinal Ca^{2+} absorption (Sergeev and Spirichev, 1987) and osteopenia (Kabitskaya et al., 1984; Sergeev et al., 1987). Prophylactic treatment with vitamin D_3 active metabolites prevented bone loss to a significant extent in rats during long-term hypokinesia (Sergeev et al., 1982a,b, 1985, 1987; Ushakov et al., 1982, 1983a,b, 1984), indicating a crucial role for the vitamin D endocrine system in regulation of bone and Ca^{2+} metabolism in modeled weightlessness.

The phenomenology of possible changes in vitamin D-mediated biological responses (*e.g.*, calbindins) during and after space flights remains currently unknown. It seems probable that the vitamin D hormone-mediated regulation, particularly that of calbindins, may be a critical factor in adaptational and readaptational changes of at least Ca^{2+} metabolism under the action of weightlessness/gravity.

We participated in the organ sharing program for tissues from rats flown aboard the Spacelab 3 mission and compared the effects of microgravity on calbindins in rats in space *vs.* "grounded" animals (synchronous Animal Enclosure Module, tail suspension, and vivarium controls). We hypothesized that exposure to microgravity might affect expression of vitamin D-dependent calcium binding proteins, calbindin-D_{28k} and calbindin-D_{9k}. To test this hypothesis we evaluated the following parameters in the kidney and intestine: 1) calbindin-D_{28k} and calbindin-D_{9k} contents; 2) immunocytochemical expression of calbindin-D_{28k} and calbindin-D_{9k}; 3) the level of vitamin D receptors.

MATERIALS AND METHODS

Sample handling

Male Sprague-Dawley rats (weighing ca. 150 g and aged 6 wk at launch) were flown for 9 days aboard the Spacelab 3 mission. Upon return to earth, the animals were dissected within "zero", 24 and 72 h post-flight (groups FR0, FR24, and FR72). Age- and sex-matched ground control animals were maintained in the Animal Enclosure Module (AEM) where factors of the space flight, except microgravity, were synchronously reproduced (groups FCR0, FCR24, and FCR72). Tail suspension rats were used as a model which mimics some effects of microgravity (groups SynSuspR0, SynSuspR24, and SynSuspR72); corresponding controls for these animals were rats kept in the vivarium (SynVivR0, SynVivR24, and SynVivR72). Moreover, the pre-flight, basal control group (VivL0) was dissected before launch.

The organs (right kidney and the washed upper portion of the small intestine ca. 10 cm in length) were snap-frozen in liquid nitrogen, stored at -70°C and shipped to the laboratory on dry ice. Left kidney, duodenum and pancreas were fixed in formalin.

Enzyme-linked immunosorbent assay

Calbindin-D_{28k} and calbindin-D_{9k} in kidneys and calbindin-D_{9k} in intestine were measured by means of an enzyme-linked immunosorbent assay (ELISA), as described previously (Miller and Norman, 1983; Rhoten and Sergeev, 1994). Diluted cytosol aliquots (50 μL; 0.5 mg/mL total protein of kidney cytosol for calbindin-D_{28k}, 0.1 mg/mL total protein of kidney cytosol for calbindin-D_{9k}, and 0.1 mg/mL total protein of intestinal cytosol for calbindin-D_{9k}) were assayed in calbindin-D_{28k}-coated (10 ng/well) or calbindin-D_{9k}-coated (2.5 ng/well) multiwell flat bottomed immunoassay plates. Chicken intestinal calbindin-D_{28k} was a gift from Dr. A.W. Norman (University of California-Riverside), and bovine intestinal calbindin-D_{9k} was purchased from Sigma (St. Louis, MO). Calbindin-coated plates were washed and preblocked with 1%

bovine serum albumin, 0.5% Tween 20 in phosphate buffered saline (PBS), and then incubated for 2 h at room temperature with unknown, standards (0 - 400 ng calbindin-D_{28k}/well or 0 - 12.5 ng calbindin-D_{9k}/well) and primary antibody (150 μL; mouse monoclonal anti-calbindin-D_{28k}, clone CL-300, Sigma, 1:140,000 dilution, or rabbit antiserum against calbindin-D_{9k}, 1:5,000 dilution, a gift from Dr. M.E. Bruns). The washed plates were then incubated for 2 h with a secondary antibody (alkaline phosphatase labeled goat anti-mouse or anti-rabbit IgG, 1:1,500 dilution, Sigma). Substrate, *p*-nitro-phenylphosphate (1 mg/mL in diethanolamine buffer, pH 9.8), was used to produce a chromogen which was quantitated at 405 nm in the microplate reader. Protein concentration in cytosols was measured with a Bio-Rad detergent-compatible protein microassay (Bio-Rad Laboratories, Hercules, CA), using a microplate format.

Immunocytochemistry

Cellular localization and quantification of calbindin-D_{28k} in kidneys, calbindin-D_{9k} in duodenum, and insulin in pancreas was carried out on the formalin-fixed tissues, as described previously (Rhoten et al., 1985; Rhoten, 1987; Rhoten and Christakos, 1990). Fixed tissues were embedded in Paraplast (Monoject Scientific, St. Louis, MO). Microtome sections were affixed to Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA) and stored at room temperature until use. Paraffin was removed and sections rehydrated. Slides were treated with 3% H_2O_2 in PBS for 10 min, rinsed in PBS, and then incubated overnight in a humid chamber at 4°C with primary antibody. The antibodies used were mouse anti-calbindin-D_{28k}, clone CL-300, 1:200 dilution in Tris buffered saline, pH 7.6, containing 2% normal goat serum, and 1% albumin (SA-TBS); rabbit anti-rat intestinal calbindin-D_{9k}, 1:400 dilution in SA-TBS; and guinea pig anti-insulin, 1:200 dilution in SA-TBS. Slides were then washed with SA-TBS, and primary antibodies detected using goat peroxidase labeled anti-mouse IgG, 1:100 dilution (Sigma); goat peroxidase labelled anti-rabbit IgG, 1:100 dilution (Incstar, Stillwater, MN); and rat peroxidase labelled anti-guinea pig IgG, 1:200 dilution (Sigma). Slides were incubated with secondary antibodies for 45 min at room temperature. Chromogen used was 3,3'-diaminobenzidine.

In situ levels of the calbindins and insulin were quantified on the basis of the intensity

of the oxidized diaminobenzidine reaction product present in individual cells, using an Image-1 image acquisition, processing and analysis system (Universal Imaging, West Chester, PA). Labeling intensity (brightness) was measured on digitized images in arbitrary O.D. units based on a 255 tone gray scale, where the value of zero is completely black and 255 is completely white (transparent). The intensity value encompassed both the number of labeled cells and their individual brightness values, and was obtained by defining the outline of the cell cluster and determining the average brightness value over the entire area. The same defined area was placed on the image close to the measured positive (darker) area to determine the level of background non-specific staining. Data are expressed as relative labeling intensity in %, *i.e.*, (brightness of the positive area)/(brightness of the background area) X 100, so that lower (*i.e*, more dense) numbers correspond to higher levels of calbindins. For each stained slide, 2 - 3 fields of cells were captured and at least three areas of labeled cells were counted.

Western blot analysis

Vitamin D receptors in the intestine and kidney were detected using Western blot technique. Tissues were thawed on ice, intestinal mucosa was scraped, and homogenates (20% wt/vol) in a high-ionic-strength buffer (KTED: 300 mM KCl, 10 mM Tris, pH 7.4, 1.5 mM EDTA, 5.0 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) were prepared (Sergeev and Norman, 1992; Norman et al., 1993). The cytosol fraction of homogenates (35,000 *g*, 1 h, 4°C) was used for Western blot analysis.

The cytosol aliquots (150 μg protein) were subjected to SDS-polyacrylamide (12%) gel electrophoresis and transfered to nitrocellulose sheets using a Bio-Rad transfer unit. The sheets were preblocked with 5% non-fat milk in PBS and then probed with rat monoclonal anti-VDR antibodies (1:500 dilution, 2 h at room temperature) (Affinity BioReagents, Golden, CO). The bands were visualized with alkaline phosphatase labeled secondary antibodies (anti-rat IgG, Sigma) for 1 h at room temperature. Chromogen used was BCIP/NBT.

For Western blot analysis of calbindin-D_{28k}, kidney cytosols were subjected to SDS-PAGE, transfer and blocking, as described for VDRs. The nitrocellulose sheets were probed with mouse

monoclonal anti-calbindin-D_{28k} (1:200 dilution, 2 h at room temperature), and the bands were visualized with alkaline phosphatase labeled secondary antibodies (anti-mouse IgG, Sigma) (Mutema and Rhoten, 1994).

In Western blot analysis of β-actin, primary antibodies used were mouse monoclonal anti-β-actin (Clone Ac-15, 1:1000 dilution; Sigma) and secondary antibodies were alkaline phosphatase labeled anti-mouse IgG (1:1000 dilution; Sigma).

Statistics

Statistical analysis of the data was performed using Sigma Stat v. 1.0 software (Jandel Scientific, San Rafael, CA).

RESULTS

Measurement of calbindin-D_{28k} and calbindin-D_{9k} in kidneys and calbindin-D_{9k} in intestine by ELISA

ELISA was used to measure total calbindin contents in kidneys and the intestinal mucosa of space, suspension and ground animals. Calbindin contents varied among the individual animals (see Appendix). However, when data were pooled and normalized per mg protein, the calbindin-D_{28k} content in kidneys and calbindin-D_{9k} content in the intestine of space and suspension animals proved to be significantly reduced compared with ground control animals (Fig. 1). In terms of the flight groups, this decrease was 24.0, 23.9 and 23.5% for calbindin-D_{28k} in kidneys at "zero", 24 and 72 h post-flight, respectively (see also Fig. 1, *left panel*); for calbindin-D_{9k} in the intestine the decrease was 57.8, 19.2 and 47.3% at "zero", 24 and 72 h post-flight (see also Fig. 1, *middle panel*). Quantitatively similar decreases were revealed when synchronous suspension and vivarium groups were compared at "zero", 24 and 72 h post-flight (see Fig. 1).

The content of calbindin-D_{9k} in kidneys was very low, as compared with the calbindin-D_{28k} content (in ng/mg protein range *vs.* μg/mg protein range for calbindin-D_{28k}) (Fig. 1, *right panel*). An apparent decrease in the renal calbindin-D_{9k} of suspension animals at "zero" and 72 h, but not 24 h post-flight was observed. This trend was statistically significant for FR0 *vs.* FCR0 groups.

In situ quantification of calbindin-D_{28k} in kidneys, calbindin-D_{9k} in intestine and insulin in pancreas using immunocytochemistry

Immunocytochemistry (ICC) in combination with quantitative computer image analysis was used to measure *in situ* the expression of calbindins in kidneys and intestine, and insulin in pancreas. The predominate immunolocalization of renal calbindin-D_{28k} in all animals was similar to that first reported by us (Rhoten and Christakos, 1981), *i.e.*, cells of distal convoluted tubules, connecting tubules and cortical collecting tubules. Some flight and grounded animals

had kidneys exhibiting a highly variable amount of immunoreactivity for calbindin-D_{28k} in the medullary collecting ducts and papillary ducts. The absence of consistent immunolocalization in medullary collecting ducts and papillary ducts argued against quantifying the calbindin-D_{28k} found in these sites. Intestinal localization of calbindin-D_{9k} appeared to be confined to the absorptive cells. As seen in Fig. 2, there was a large decrease in the distal tubular cell-associated calbindin-D_{28k} immunoreactivity and in the absorptive cell-associated calbindin-D_{9k} immunoreactivity in the space kidneys and intestine, as compared with matched ground control animals. Insulin was localized to relatively large numbers of cells making up the core of pancreatic islets. A result consistent with the localization of insulin in β-cells of the rat. There was no consistent difference in pancreatic insulin immunoreactivity of space and ground animals (see Fig. 2). No specific immunoreactivity for calbindin-D_{28k} was observed in the pancreatic islet cells of any of the flight or grounded animals.

Summary of quantitative image analysis of tissues from space, suspension and ground animals are presented in Fig. 3. Comparison of groups was done as described above for ELISA. Reductions in the calbindin-D_{28k} level in kidneys and the calbindin-D_{9k} level in the intestine were similar to those found with ELISA (see Fig. 3, *left* and *middle panels*). Linear regression analysis of data obtained using ELISA and quantitative ICC, showed a statistically significant correlation between two methods (Fig. 4). Insulin level in pancreas varied widely among animals within a group, and no apparent trend to the decreased insulin immunoreactivity was revealed in space and suspension animals (see Fig. 3, *right panel*).

Detection of vitamin D receptors in the intestine and kidneys using Western blot analysis

VDR levels in kidneys and the intestinal mucosa of flight and flight control animals were compared using Western blotting with rat monoclonal anti-VDR antibodies. As seen on representative immunoblots (Fig. 5), there were no consistent changes in the VDR level, based on the intensity of specific bands. Importantly, there was also no changes in the intestinal β-actin level. Calbindin-D_{28k} levels, measured on some of the same membranes, varied in the same way as seen quantitatively by ELISA.

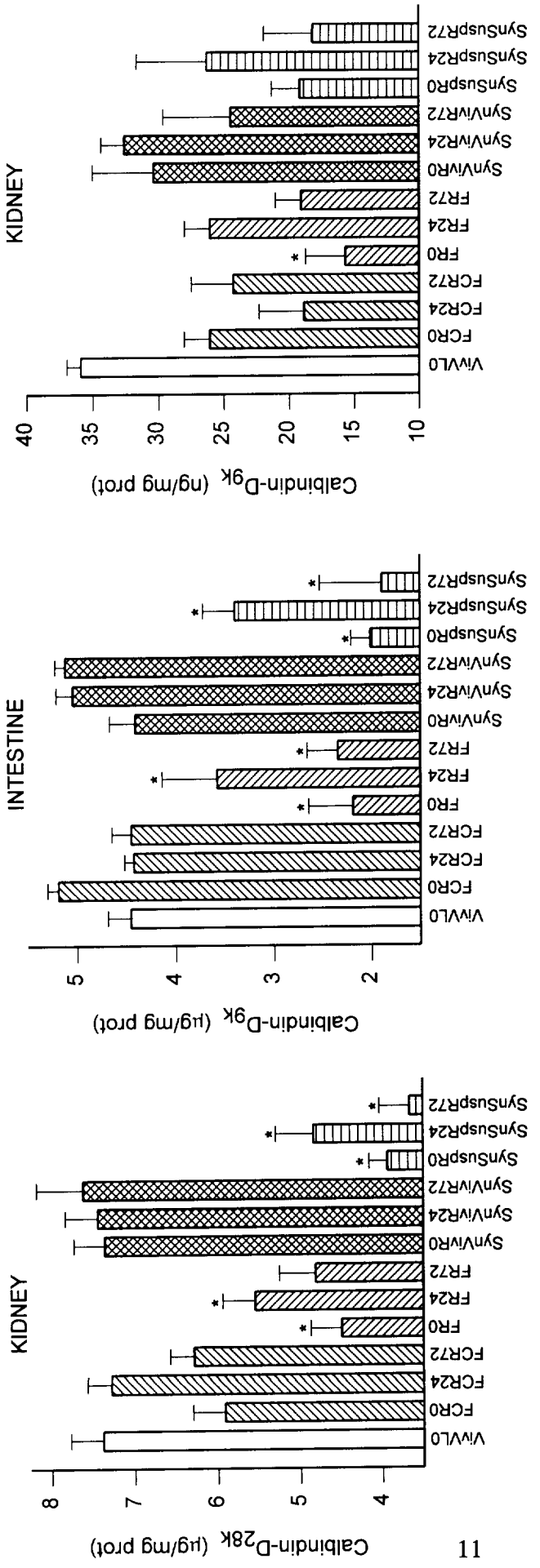
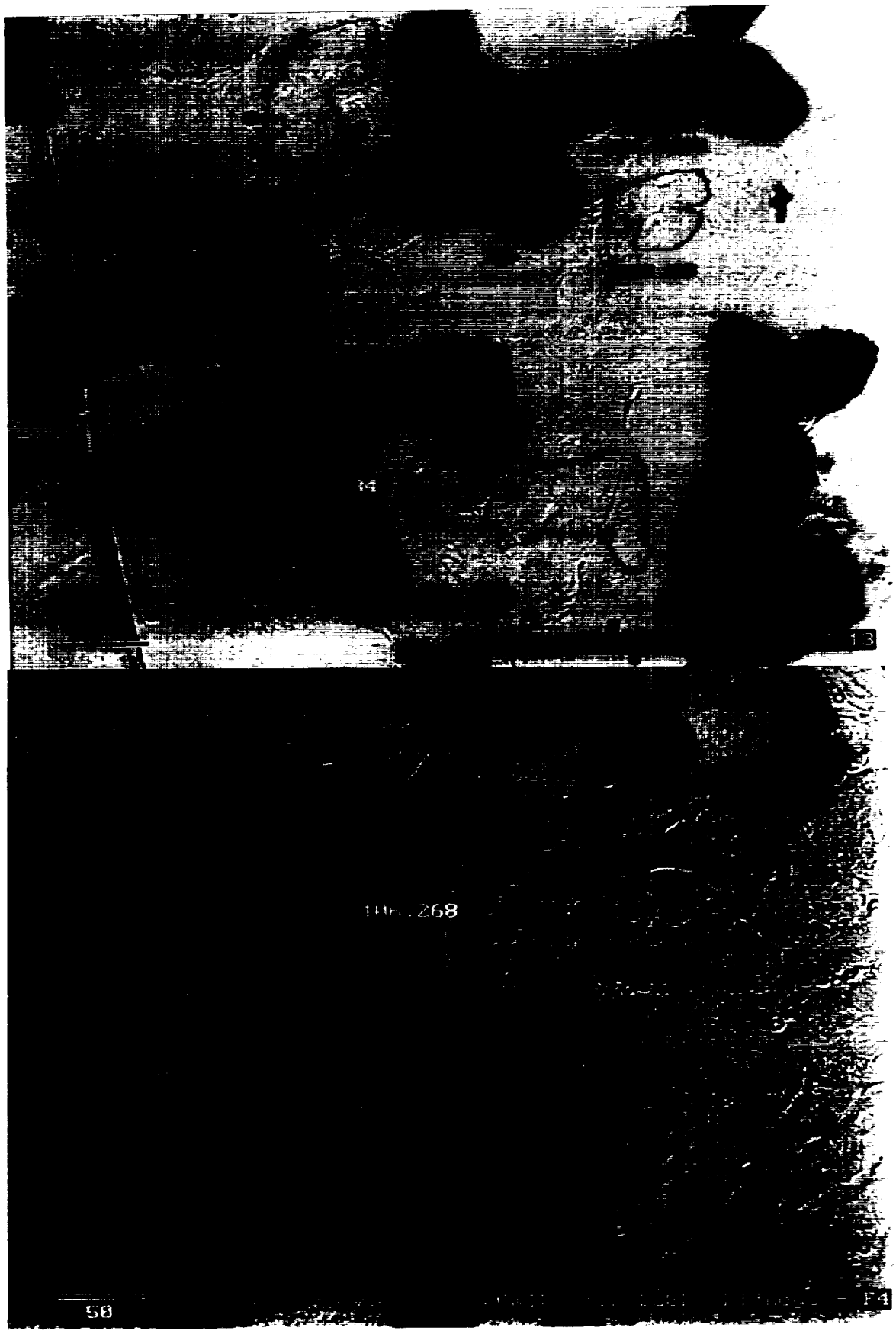
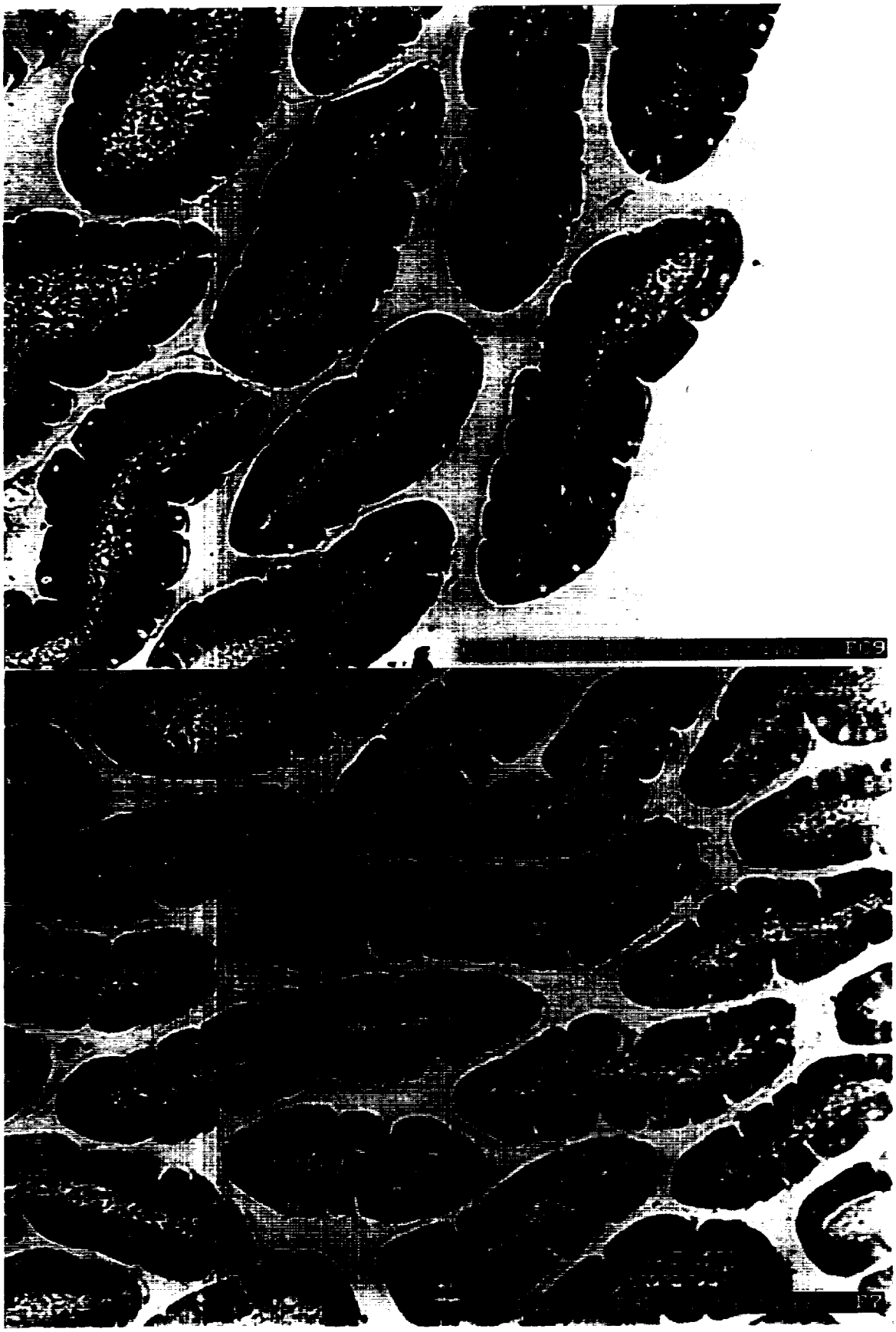


Fig. 1. Calbindin contents in the intestine and kidneys of space, suspension and ground animals. Calbindin-D_{28k} and calbindin-D_{9k} were measured by ELISA, as described in *Materials and Methods*. Group abbreviations are also presented in *Materials and Methods*. The data, analyzed by ANOVA, represent mean values ± SEM. Only significance of differences between F *vs.* FC and SynSusp *vs.* SynViv groups are presented. (*), $P < 0.05$.





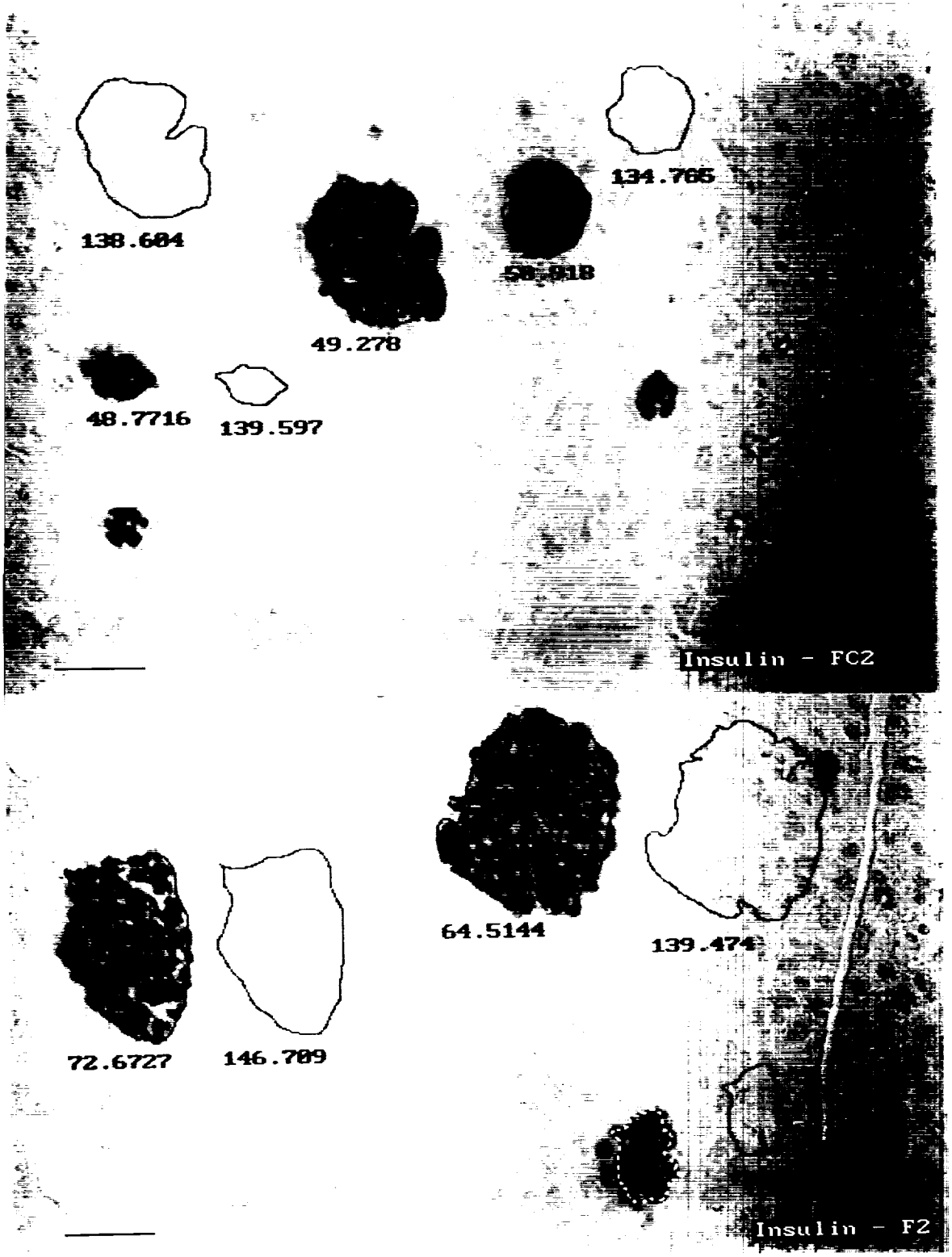


Fig. 2. Immunocytochemical localization and quantification of calbindins and insulin. Tissue sections were immunoreacted and calbindin-D_{28k} in kidney *(p. 12)*, calbindin-D_{9k} in the intestine *(p. 13)*, and insulin in pancreas *(p.14)* were measured, as described in *Materials and Methods*. Scale marker = 50 μm (kidneys, pancreas) or 100 μm (intestine).

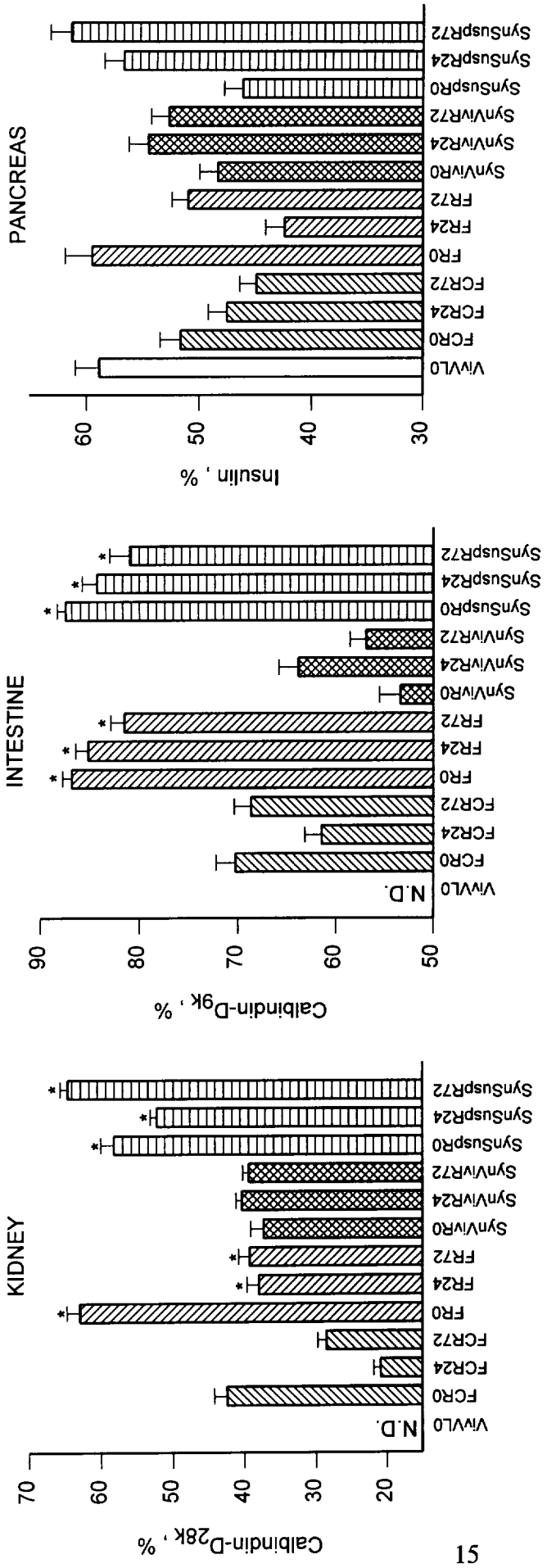


Fig. 3. Calbindin levels in the intestine and kidneys, and insulin level in pancreas of space, suspension and ground animals. Calbindin-D_{28k} and calbindin-D_{9k} were quantified by ICC, as described in *Materials and Methods*. Note that higher (*i.e.*, less dense) values correspond to lower levels of calbindins and insulin (see also *Materials and Methods*). The data, analyzed by ANOVA, represent mean values ± SEM. Only significance of differences between F *vs.* FC and SynSusp *vs.* SynViv groups are presented. (*), $P < 0.05$; N.D., not determined.

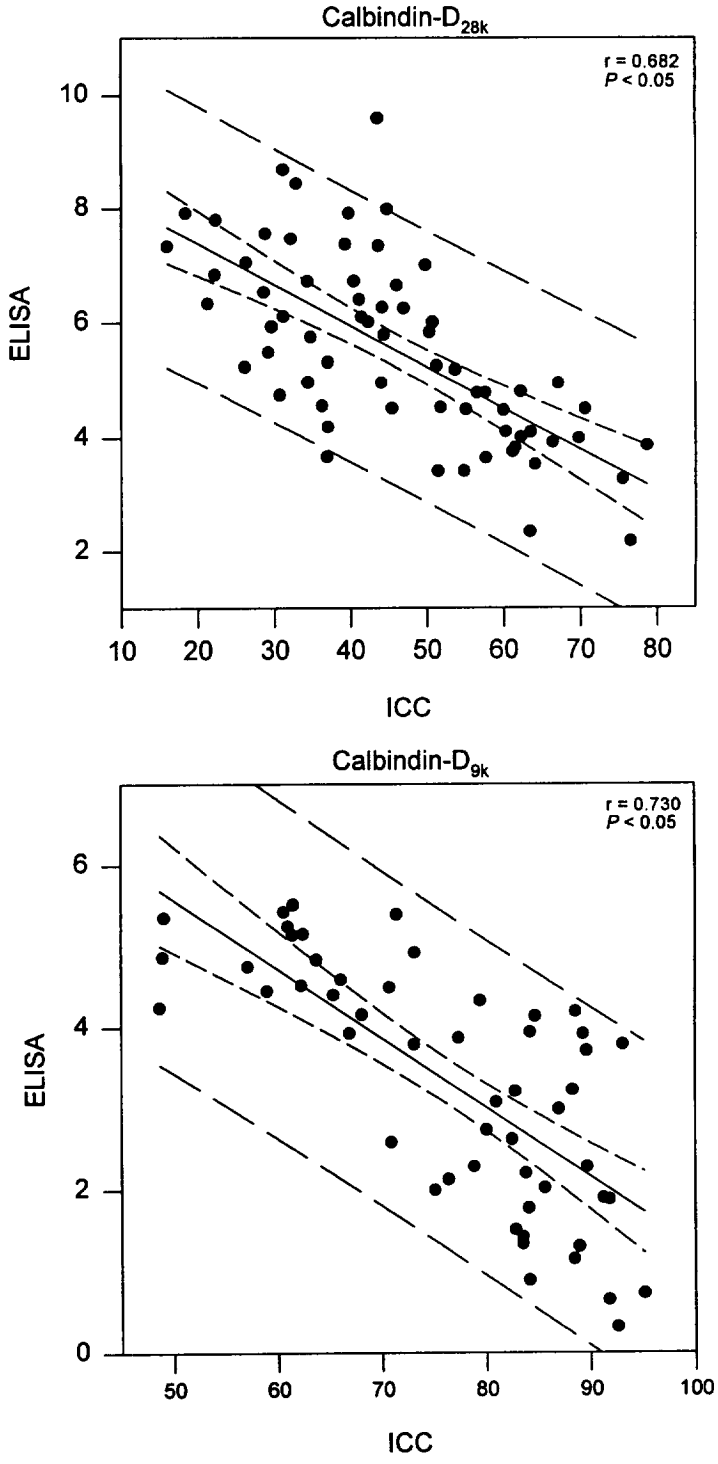


Fig. 4. Correlation between enzyme-linked immunosorbent and immunocytochemical quantification of renal calbindin-D_{28k} and intestinal calbindin-D_{9k}. Linear regression analysis revealed for two methods, a correlation coefficient of ≥ 0.7 and $P < 0.05$. A 95% confidence interval is shown by short-dashed lines; long-dashed lines fit values predicted by the regression model.

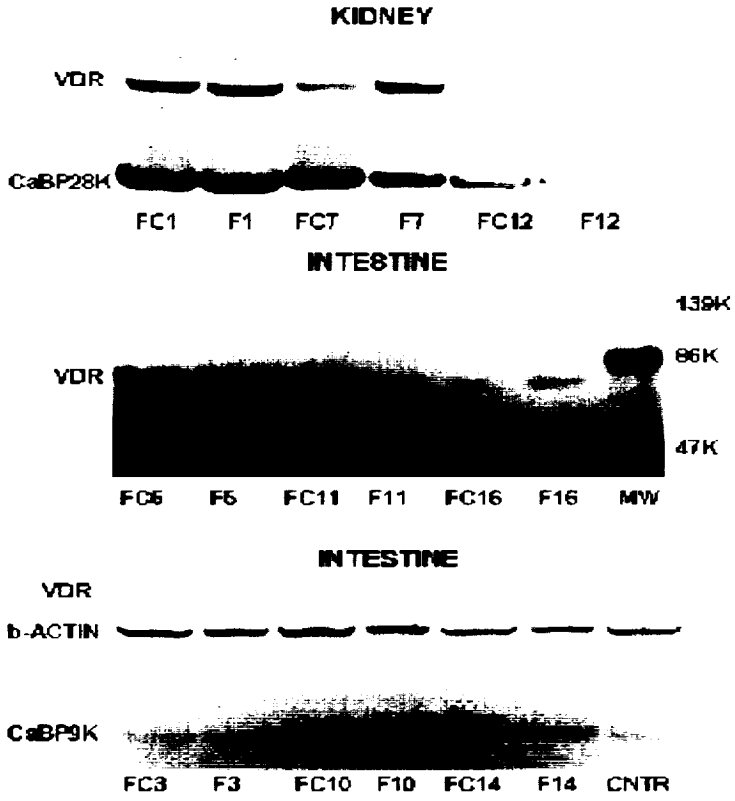


Fig. 5. Western blot analysis of VDRs and calbindins in the intestine and kidneys of space and ground animals. The cytosol extracts of the intestinal mucosa and kidneys were subjected to SDS-PAGE and immunoblotting, as described in *Materials and Methods*. Protein standards of the indicated molecular masses (K) were run in a parallel lane. CNTR = cytosol extract of MDBK cells which have lower levels of the VDR expression, as compared with rat tissues.

DISCUSSION

Calcium metabolism and its regulation change promptly (within days) with exposure of the body to microgravity or simulated weightlessness (hypokinesia, suspension) (Arnaud and Morey-Holton, 1989; Spirichev and Sergeev, 1988). The vitamin D hormone, $1,25(OH)_2D_3$, is the critical component of the Ca^{2+}-regulating endocrine system (Norman et al., 1982, 1992). Vitamin D-dependent Ca^{2+}-binding proteins, calbindin-D_{28k} and calbindin-D_{9k}, play the essential roles in the regulation of Ca^{2+} metabolism and maintenance of cellular Ca^{2+} homeostasis. Calbindins are involved in the intestinal Ca^{2+} absorption, renal Ca^{2+} reabsorption, and intracellular Ca^{2+} buffering (Christakos et al., 1989; Rhoten and Sergeev, 1994; Johnson and Kumar, 1994).

In this study, we have demonstrated, using two independent approaches, a significant decrease in the renal calbindin-D_{28k} and intestinal calbindin-D_{9k} content and immunocytochemical expression in rats exposed for 9 days to microgravity on board the Spacelab 3 mission. Importantly, suspension animals demonstrated virtually identical changes in calbindins. These findings strongly suggest that factors of modelled weightlessness (suspension) can mimic the effects of microgravity on calbindins.

Because calbindins are vitamin D-regulated proteins and because VDRs seem to be not affected by the factors of space flight, the decreased circulating concentration of the hormonal form of vitamin D, $1,25(OH)_2D_3$, might be primarily responsible for the reduction in calbindin contents and levels of expression in kidneys and intestine of flight and suspension animals. Decreased production of $1,25(OH)_2D_3$ in kidneys may determine, to a large extent, such reduction in the serum $1,25(OH)_2D_3$ concentration. As we have shown earlier, this is the case for hypokinetic model of weightlessness in rats (Sergeev et al., 1984; Spirichev and Sergeev, 1988).

The depression of calbindin-D_{28k} in kidneys and calbindin-D_{9k} in the intestine appears to be relatively selective, because β-actin level in the intestine and insulin level in the pancreas, evaluated by Western blot analysis and ICC respectively, were not changed in space and

suspension animals.

It is also noteworthy, that the decrease in calbindins was independent of the time of harvesting the tissues after reentry. This suggests that the reduction in amounts of calbindins and gene expression for these calcium-binding proteins is a long-lasting effect of microgravity and suspension *per se*, rather than a rapid, transient stress response.

Functional consequences of decreased calbindin expression, at the organismal level, might be the decreased absorption of Ca^{2+} in the intestine, increased excretion of Ca^{2+} in the urine, and, at the cellular level, the sustained increase in the concentration of cytosolic free Ca^{2+}, which may interfere with Ca^{2+} signaling and cause an increase in the rate of cell death.

Thus, our study implies that the decrease of calbindin contents and immunocytochemical expressions in kidneys and intestine from rats exposed to microgravity and modelled weightlessness (tail suspension) may be directly related to changes in Ca^{2+} metabolism under the effects of these conditions. Changes in calbindins may be attributed to an interference of microgravity and suspension with functioning of the vitamin D-endocrine system. Future space and ground-based experiments are necessary to test this hypothesis.

CONCLUSIONS

Content and immunocytochemical expression of calbindin-D_{28k} in kidney and calbindin-D_{9k} in the intestine were decreased in flight *vs.* flight control animals and in tail suspension *vs.* vivarium controls at "zero", 24 and 72 h post-flight. Decrease in renal calbindin-D_{28k} and intestinal calbindin-D_{9k} may affect Ca^{2+} handling in these organs; namely, it may be partly responsible for the increased Ca^{2+} excretion in the urine and decreased Ca^{2+} absorption in the small intestine observed after a short-term space flight and modelled weightlessness.

No consistent changes in the low levels of intestinal and renal VDRs of flight animals were found with Western blot analysis, implying that decreased circulating concentration of $1,25(OH)_2D_3$ in space flight and modelled weightlessness might reduce the calbindin expression in these tissues.

Factors of space flight and modelled weightlessness had no apparent effect on the immunocytochemical expression of insulin in pancreas.

Our findings suggest that the decreased expression of calbindins after a short-term exposure to microgravity and modelled weightlessness may affect cellular Ca^{2+} homeostasis and contribute to Ca^{2+} and bone metabolism disorders induced by space flight.

SIGNIFICANCE AND FUTURE GOALS

This investigation has allowed us to assess the effects of short-term space flight on calbindins and their regulation by the vitamin D endocrine system. Assessment included the application of a new method for quantifying calbindins at the single cell level. This technically significant advance should be utilized in future studies on cells, tissues and organs in altered gravity states. The results contribute to our understanding of the genesis of space flight-induced disorders of Ca^{2+} metabolism.

Future space and ground-based experiments are necessary to study functional consequences of the decreased expression of calbindins at the cellular level and to investigate whether up-regulation of calbindins, *e.g.*, with analogs of the vitamin D hormone will be useful for prevention of disorders of Ca^{2+} metabolism induced by space flight.

ACKNOWLEDGEMENTS

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Part of this study was presented at the 26th Congress of the Anatomical Society of Southern Africa, South Africa, 22 April, 1996. Some of the results will also be presented at the Annual Scientific Congress of the Zimbabwe Association of Clinical Pathologists and Medical Scientists, 6 July, 1996.

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APPENDIX

Calbindin-D28k, Kidney, ELISA

Descriptive Statistics:

Column	Size	Mean
VivCL0	6	7.38
FCR0	6	5.92
FCR24	5	7.29
FCR72	5	6.29
FR0	6	4.50
FR24	5	5.55
FR72	5	4.81
SynVivR0	6	7.37
SynVivR24	5	7.45
SynVivR72	5	7.62
SynSuspR0	11	3.93
SynSuspR24	5	4.84
SynSuspR72	5	3.66

Column	Std Dev	Std. Error	Range
VivCL0	0.961	0.392	2.69
FCR0	0.943	0.385	2.52
FCR24	0.639	0.286	1.59
FCR72	0.645	0.289	1.59
FR0	0.925	0.378	2.73
FR24	0.876	0.392	2.08
FR72	0.980	0.438	2.59
SynVivR0	0.913	0.373	2.75
SynVivR24	0.884	0.395	2.35
SynVivR72	1.256	0.562	3.20
SynSuspR0	0.748	0.225	2.78
SynSuspR24	1.028	0.460	2.83
SynSuspR72	0.822	0.367	2.17

Column	Max	Min	Median
VivCL0	9.01	6.33	7.01
FCR0	7.78	5.26	5.65
FCR24	7.92	6.33	7.33
FCR72	6.83	5.25	6.53
FR0	6.01	3.28	4.51
FR24	6.65	4.57	5.76
FR72	6.26	3.67	4.97
SynVivR0	8.69	5.94	7.38
SynVivR24	8.44	6.10	7.37
SynVivR72	9.60	6.40	7.47
SynSuspR0	4.95	2.17	4.00
SynSuspR24	6.25	3.42	4.79
SynSuspR72	4.51	2.34	3.83

Calbindin-D9k, Intestine, ELISA

Descriptive Statistics:

Column	Size	Mean
VivCL0	6	4.46
FCR0	6	5.19
FCR24	5	4.43
FCR72	5	4.46
FR0	6	2.19
FR24	5	3.58
FR72	5	2.35
SynVivR0	6	4.42
SynVivR24	5	5.05
SynVivR72	5	5.12
SynSuspR0	11	2.01
SunSuspR24	5	3.40
SynSuspR72	5	1.89

Column	Std Dev	Std. Error	Range
VivCL0	0.563	0.2299	1.447
FCR0	0.271	0.1106	0.665
FCR24	0.215	0.0960	0.594
FCR72	0.438	0.1958	1.216
FR0	1.115	0.4552	2.811
FR24	1.264	0.5652	2.992
FR72	0.703	0.3146	1.777
SynVivR0	0.632	0.2582	1.821
SynVivR24	0.367	0.1641	0.924
SynVivR72	0.224	0.1001	0.561
SynSuspR0	0.651	0.1962	2.261
SunSuspR24	0.728	0.3257	1.733
SynSuspR72	1.428	0.6387	3.473

Column	Max	Min	Median
VivCL0	5.06	3.618	4.54
FCR0	5.50	4.833	5.26
FCR24	4.75	4.157	4.45
FCR72	5.14	3.926	4.40
FR0	3.71	0.895	2.09
FR24	4.33	1.335	4.13
FR72	3.08	1.302	2.62
SynVivR0	5.36	3.535	4.38
SynVivR24	5.52	4.591	4.99
SynVivR72	5.43	4.866	5.12
SynSuspR0	2.99	0.730	2.02
SunSuspR24	3.94	2.204	3.78
SynSuspR72	3.79	0.315	2.13

Calbindin-D9k, Kidney, ELISA

Descriptive Statistics:

Column	Size	Mean
VivCL0	6	35.9
FCR0	6	26.1
FCR24	5	18.9
FCR72	5	24.3
FR0	6	15.7
FR24	5	26.1
FR72	5	19.1
SynVivR0	6	30.4
SynVivR24	5	32.6
SynVivR72	5	24.5
SynSuspR0	11	19.2
SunSuspR24	5	26.3
SynSuspR72	3	18.2

Column	Std Dev	Std. Error	Range
VivCL0	2.55	1.04	6.90
FCR0	4.82	1.97	15.19
FCR24	7.71	3.45	17.98
FCR72	7.15	3.20	15.68
FR0	7.51	3.07	20.49
FR24	4.36	1.95	10.66
FR72	4.37	1.95	11.29
SynVivR0	11.32	4.62	31.30
SynVivR24	3.95	1.76	10.46
SynVivR72	11.52	5.15	28.37
SynSuspR0	7.16	2.16	21.47
SunSuspR24	11.96	5.35	26.42
SynSuspR72	6.50	3.75	11.78

Column	Max	Min	Median
VivCL0	40.7	33.78	35.5
FCR0	33.8	18.59	26.3
FCR24	29.0	10.99	21.3
FCR72	30.2	14.48	29.0
FR0	29.5	9.04	13.8
FR24	29.8	19.15	28.5
FR72	25.7	14.41	17.7
SynVivR0	48.5	17.19	31.3
SynVivR24	37.8	27.30	33.5
SynVivR72	44.0	15.59	21.7
SynSuspR0	27.6	6.11	23.3
SunSuspR24	40.2	13.78	31.2
SynSuspR72	25.7	13.92	15.0

Calbindin-D28k, Kidney, ICC

Descriptive Statistics:

Column	Size	Mean
FCR0	102	42.4
FCR24	50	20.9
FCR72	50	28.5
FR0	119	63.0
FR24	50	38.0
FR72	50	39.3
SynVivR0	42	37.4
SynVivR24	50	40.4
SynVivR72	50	39.4
SynSuspR0	170	59.1
SynSuspR24	50	52.3
SynSuspR72	49	64.7

Column	Std Dev	Std. Error	Range
FCR0	11.60	1.222	40.9
FCR24	6.68	0.996	28.2
FCR72	8.26	1.232	35.3
FR0	13.09	1.260	55.7
FR24	11.08	1.651	48.0
FR72	10.34	1.541	42.3
SynVivR0	10.47	1.745	35.8
SynVivR24	5.51	0.822	25.8
SynVivR72	5.86	0.874	26.8
SynSuspR0	8.83	0.714	43.8
SynSuspR24	6.05	0.902	23.7
SynSuspR72	7.01	1.058	31.8

Column	Max	Min	Median
FCR0	63.6	22.77	38.8
FCR24	37.7	9.58	19.3
FCR72	52.1	16.74	27.4
FR0	88.5	32.83	61.1
FR24	63.1	15.18	36.9
FR72	64.1	21.82	38.6
SynVivR0	60.0	24.15	32.8
SynVivR24	51.6	25.84	39.9
SynVivR72	48.6	21.82	41.2
SynSuspR0	79.1	35.33	58.5
SynSuspR24	65.2	41.44	51.0
SynSuspR72	78.1	46.30	66.2

Calbindin-D9k, Intestine, ICC

Descriptive Statistics:

Column	Size	Mean
FCR0	28	70.2
FCR24	21	61.4
FCR72	28	68.6
FR0	42	86.8
FR24	35	85.2
FR72	35	81.5
SynVivR0	21	53.4
SynVivR24	14	63.8
SynVivR72	21	56.9
SynSuspR0	56	87.5
SynSuspR24	35	84.3
SynSuspR72	35	81.0

Column	Std Dev	Std. Error	Range
FCR0	9.79	1.999	33.8
FCR24	7.30	1.721	31.3
FCR72	8.36	1.707	30.5
FR0	5.45	0.909	23.4
FR24	6.79	1.239	27.0
FR72	7.46	1.362	35.1
SynVivR0	9.00	2.121	33.3
SynVivR24	6.89	1.990	26.1
SynVivR72	7.00	1.650	26.3
SynSuspR0	5.91	0.853	24.8
SynSuspR24	8.19	1.495	36.2
SynSuspR72	11.01	2.010	44.4

Column	Max	Min	Median
FCR0	84.3	50.5	69.8
FCR24	77.2	45.9	61.4
FCR72	87.2	56.7	71.6
FR0	94.6	71.2	87.3
FR24	95.5	68.5	85.5
FR72	96.2	61.1	80.9
SynVivR0	71.4	38.1	52.7
SynVivR24	76.6	50.6	63.4
SynVivR72	68.8	42.5	58.3
SynSuspR0	98.3	73.5	87.4
SynSuspR24	100.0	63.8	84.3
SynSuspR72	101.2	56.7	80.2

Insulin, pancreas, ICC

Descriptive Statistics:

Column	Size	Mean
VivCL0	23	58.8
FCR0	25	51.7
FCR24	24	47.5
FCR72	25	44.9
FR0	22	59.5
FR24	27	42.4
FR72	24	51.0
SynVivR0	26	48.3
SynVivR24	23	54.5
SynVivR72	23	52.7
SynSuspR0	55	46.1
SynSuspR24	25	56.7
SynSuspR72	24	61.3

Column	Std Dev	Std. Error	Range
VivCL0	9.16	2.10	25.7
FCR0	8.12	1.82	29.1
FCR24	7.23	1.66	22.8
FCR72	6.59	1.47	24.7
FR0	9.90	2.40	28.6
FR24	8.09	1.72	30.4
FR72	6.32	1.45	24.4
SynVivR0	7.43	1.62	30.4
SynVivR24	7.30	1.72	28.5
SynVivR72	6.61	1.56	22.1
SynSuspR0	11.00	1.66	45.6
SynSuspR24	7.65	1.71	25.4
SynSuspR72	8.10	1.86	31.8

Column	Max	Min	Median
VivCL0	69.1	43.4	60.9
FCR0	66.7	37.7	52.0
FCR24	59.9	37.1	46.9
FCR72	55.7	31.0	45.8
FR0	71.3	42.7	64.3
FR24	59.2	28.8	41.6
FR72	59.3	34.9	52.9
SynVivR0	62.0	31.7	47.0
SynVivR24	66.7	38.1	56.0
SynVivR72	64.2	42.1	52.7
SynSuspR0	66.2	20.6	44.6
SynSuspR24	69.3	43.9	58.0
SynSuspR72	75.8	44.0	61.8

	S-Code	K28k-ELISA	K28k-ICC	S'-Code	K9k-ELISA
1	FC1	5.7992	44.3795	FC1	26.2549
2	FC2	5.8437	50.3239	FC2	26.2549
3	FC3	5.2572	51.2744	FC3	18.5879
4	FC4	5.3259	37.0121	FC4	26.3246
5	FC5	5.4958	29.2355	FC5	25.3488
6	FC6	7.7772	n.d.	FC6	33.7825
7	FC7	7.8015	22.3640	FC7	10.9906
8	FC8	7.0531	26.3569	FC8	11.1997
9	FC9	7.3322	15.9961	FC9	28.9732
10	FC10	7.9228	18.3775	FC10	21.3062
11	FC11	6.3331	21.3141	FC11	21.8638
12	FC12	6.1106	31.2181	FC12	14.4756
13	FC13	6.8347	22.2387	FC13	18.8667
14	FC14	6.7214	34.3839	FC14	30.1581
15	FC15	6.5313	28.6618	FC15	29.0429
16	FC16	5.2450	26.1378	FC16	28.9732
17	F1	3.2751	75.5400	F1	9.0390
18	F2	3.8657	78.6997	F2	17.8212
19	F3	6.0095	60.7473	F3	14.9635
20	F4	4.5007	55.1229	F4	10.0845
21	F5	4.5210	45.4431	F5	12.5937
22	F6	4.8082	62.2483	F6	29.5308
23	F7	6.0136	42.3118	F7	29.8096
24	F8	6.6486	46.0619	F8	28.4853
25	F9	5.7628	34.7636	F9	24.5124
26	F10	4.5695	36.2686	F10	28.4853
27	F11	4.7515	30.7326	F11	19.1455
28	F12	4.9740	34.4122	F12	16.7757
29	F13	3.6715	36.9223	F13	20.9577
30	F14	4.1974	37.0440	F14	25.6973
31	F15	6.2644	44.1456	F15	17.6818
32	F16	4.9659	44.0181	F16	14.4059
33	V1	7.9956	n.d.	V1	33.7825
34	V2	9.0150	n.d.	V2	40.6828
35	V3	6.9844	n.d.	V3	35.1068
36	V4	7.0329	n.d.	V4	36.2917
37	V5	6.9480	n.d.	V5	35.8735
38	V6	6.3291	n.d.	V6	33.7825
39	V7	7.5588	28.8683	V7	19.4940
40	V8	8.6873	41.1512	V8	48.4892
41	V9	7.0005	49.8078	V9	30.5763
42	V10	7.7974	n.d.	V10	32.1097
43	V11	7.2109	n.d.	V11	17.1339
44	V12	5.9367	29.6742	V12	34.4795
45	V13	7.3686	39.3003	V13	34.0613
46	V14	6.0985	41.4264	V14	33.5037

47	V15	7.9875	44.8223	V15		37.7554
48	V16	7.3403	43.6272	V16		30.4369
49	V17	8.4446	32.8965	V17		27.3004
50	V18	7.4657	32.2011	V18		24.8609
51	V19	6.4019	41.1306	V19		16.4272
52	V20	7.9188	39.7683	V20		21.7244
53	V21	6.7255	40.4611	V21		15.5908
54	V22	9.5974	43.5480	V22		43.9587
55	S1	4.9538	67.1326	S1		25.9064
56	S2	3.9951	49.8078	S2		18.5879
57	S3	4.7879	56.5361	S3		23.9548
58	S4	4.1124	60.2975	S4		14.6150
59	S5	3.4167	n.d.	S5		23.3275
60	S6	2.1749	n.d.	S6		11.4785
61	S7	4.4846	n.d.	S7		6.1116
62	S8	3.7605	n.d.	S8		24.5821
63	S9	3.6472	n.d.	S9		11.5482
64	S10	3.9223	n.d.	S10		27.5792
65	S11	4.0153	n.d.	S11		23.3972
66	S12	5.1924	53.6962	S12		31.2036
67	S13	6.2482	46.9773	S13		32.7370
68	S14	4.5371	51.7776	S14		40.1949
69	S15	4.7879	57.6177	S15		13.7786
70	S16	3.4167	51.4943	S16		13.7786
71	S17	3.8333	61.5358	S17		25.6973
72	S18	2.3407	63.4326	S18		13.9180
73	S19	4.1003	63.5284	S19	n.d.	
74	S20	3.5340	64.1405	S20	n.d.	
75	S21	4.5088	70.6381	S21		15.0332

	S"-Code	I9k-ELISA	I9k-ICC	S_Code	Ins-ICC
1	FC1	5.3610	n.d.	FC1	50.2167
2	FC2	5.4985	n.d.	FC2	44.3902
3	FC3	5.3940	81.4828	FC3	59.4336
4	FC4	5.1520	62.4757	FC4	n.d.
5	FC5	4.8330	63.7480	FC5	59.8086
6	FC6	4.9210	73.2011	FC6	45.8674
7	FC7	4.4590	4.7505	FC7	47.8462
8	FC8	4.3545	4.4480	FC8	48.8582
9	FC9	4.7505	4.1565	FC9	45.9874
10	FC10	4.4480	59.0143	FC10	54.4933
11	FC11	4.1565	68.1195	FC11	38.0059
12	FC12	4.3490	n.d.	FC12	45.2896
13	FC13	2.2865	78.8619	FC13	43.8184
14	FC14	1.9015	91.2464	FC14	35.4268
15	FC15	1.1535	88.4585	FC15	50.3499
16	FC16	0.8950	84.1692	FC16	47.4671
17	F1	3.7055	89.6685	F1	65.1337
18	F2	3.2270	88.3325	F2	47.4718
19	F3	4.1895	88.6237	F3	68.2654
20	F4	3.9145	89.3571	F4	n.d.
21	F5	1.3350	83.5433	F5	62.3207
22	F6	4.3270	79.5008	F6	48.3599
23	F7	4.1345	84.7477	F7	39.1610
24	F8	1.9950	75.1093	F8	37.0330
25	F9	3.0785	81.0125	F9	48.5472
26	F10	2.6220	82.4977	F10	47.6709
27	F11	1.3020	88.9415	F11	39.2782
28	F12	2.7375	80.0568	F12	49.2748
29	F13	3.0785	81.0125	F13	51.9748
30	F14	2.6220	82.4977	F14	45.2043
31	F15	1.3020	88.9415	F15	52.9530
32	F16	2.7375	80.0568	F16	53.9826
33	V1	3.6175	n.d.	V1	68.2605
34	V2	4.3215	n.d.	V2	n.d.
35	V3	4.0630	n.d.	V3	59.7985
36	V4	4.9595	n.d.	V4	n.d.
37	V5	4.7505	n.d.	V5	57.3691
38	V6	5.0640	n.d.	V6	43.6746
39	V7	4.0300	n.d.	V7	43.7539
40	V8	4.2445	48.6879	V8	47.3921
41	V9	3.5350	n.d.	V9	n.d.
42	V10	4.8055	n.d.	V10	44.9096
43	V11	4.5195	62.3034	V11	52.9083
44	V12	5.3555	49.1160	V12	55.7019
45	V13	5.5150	61.5365	V13	57.4880
46	V14	4.9870	n.d.	V14	57.4569

47	V15	4.8385	n.d.	V15	48.0780
48	V16	5.3005	n.d.	V16	58.6382
49	V17	4.5910	66.1031	V17	50.3131
50	V18	4.9595	n.d.	V18	54.5677
51	V19	5.4270	60.6123	V19	48.0054
52	V20	4.8660	49.0113	V20	48.0726
53	V21	5.1190	n.d.	V21	61.8796
54	V22	5.2455	61.0190	V22	50.8343
55	S1	2.3250	n.d.	S1	30.1745
56	S2	2.3580	n.d.	S2	48.4928
57	S3	2.2810	89.6873	S3	59.0956
58	S4	1.5110	82.8405	S4	42.8949
59	S5	1.7750	84.1174	S5	45.5172
60	S6	2.9905	86.9966	S6	61.7201
61	S7	1.4230	83.5381	S7	42.3869
62	S8	0.7300	95.1616	S8	34.2409
63	S9	2.8310	n.d.	S9	38.6014
64	S10	2.0225	85.6282	S10	61.3123
65	S11	1.8795	91.8267	S11	42.5991
66	S12	3.2105	82.8308	S12	54.8549
67	S13	3.7825	93.1352	S13	65.2712
68	S14	3.9365	84.2747	S14	51.1843
69	S15	2.2040	83.8228	S15	61.0801
70	S16	3.8705	77.4126	S16	55.1856
71	S17	3.7880	73.1579	S17	52.7579
72	S18	2.1270	76.4148	S18	64.8716
73	S19	0.3150	92.6017	S19	71.7283
74	S20	2.5890	70.9394	S20	60.1845
75	S21	0.6530	91.7736	S21	62.0597